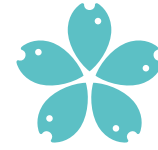Fakulta rybářství
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**Faculty of Fisheries
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Jihočeská univerzita
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**University of South Bohemia
in České Budějovice**

Crayfish as bioindicators
of water quality

Využití raků jako bioindikátorů kvality vody

WATER QUALITY GUARANTEED

Iryna Kuklina

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Czech Republic, Vodňany, 2014

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. BIOMONITORING AND BIOMONITORS

Living in the age of technological advances, the human got strongly accustomed to having most things determined and quantified. People learnt to investigate, measure and analyse even complex environmental processes. Aquatic environment is not an exception among such issues which can be studied with the aid of modern precise analytical approaches. However in spite of various advances, instead of turning to innovative tools, over and over we are studying representative aquatic organisms to find out what is really happening in underwater world. Apparently, this is because only native animals can adequately reflect ambient water conditions and signal changes associated with physico-chemical disbalance of them.

Evaluation of some physiological or behavioural functions of living organism which are changed when exposed to potentially harmful substances or certain substandard conditions is the basic principle of biological monitoring (Gunatilaka and Diehl, 2000). Biomonitoring indicates the cumulative response to overall effects in a water body – combinations of all contaminants and to other sources of environmental stress, although analytical methods can specify the exact contaminants present in the water. Nevertheless, physical and chemical analyses provide a very instant measurement (i.e. valid only for the sampling moment), while biomonitoring reflects the effects of the physico-chemical conditions to which the organisms were exposed over the time period (Chapman and Jackson, 1996).

For efficient biomonitoring of any environment, selection of proper bioindicator ("biomonitor") is critical, since it must be both sensitive enough to signal the negative conditions and tolerant enough to survive them. In most sources, bioindicators are determined as species characterising ambient conditions based on their presence (absence) and fitness (illness), often enabling to evaluate environmental state even prior to complicated measurements and laboratory analyses (Gadzała-Kopciuch et al., 2004). Particularly, an ideal bioindicator should possess the following characteristics:
- clear taxonomy,
- extensive abundance in sufficient numbers,
- simple identification and sampling,
- low dispersal rate,
- long life-span,
- high ecological importance,
- ecological and genetic stability,
- ease of maintenance in labs and experimentation,
- relatively early maturation and high reproductive rate,
- high sensitivity towards specific substances and other stressors,
- ability for bioaccumulation (Füreder and Reynolds, 2003; Gadzała-Kopciuch, et al., 2004; Kane et al., 2005).

Unlike other potential bioindicators such as fish (Petrescu-Mag et al., 2014), algae (Vincent and Kirkwood, 2014), other macroinvertebrates (Obolewski et al., 2014) etc, having certain practical disadvantages (Resh, 2008), crayfish apparently match most of the above requirements and seem to be proper biomonitors. Moreover, the crayfish state, as the consequence of environmental/anthropogenic impact, can be also screened via biochemical indicators – "biomarkers" (Demers et al., 2006; Sladkova and Kholodkevich, 2011), immunological parameters (Malev et al., 2010), through histopathological analyses (Benli, 2014), while crayfish themselves are the valuable food source for humans (Gherardi, 2011) as well as for many carnivorous representatives of aquatic fauna (El-Kholie et al., 2012).

According to changes of crayfish morpho-, etho- and physiological parameters, as well as ability to accumulate and tolerate specific substances (everything what is peculiar to bioindicators), the following biomonitoring approaches may be delineated:
-    monitoring of presence of crayfish assemblage in water bodies,
-    evaluation of accumulation of harmful substances in crayfish tissues,
-    testing of limits of crayfish tolerance to toxic compounds,
-    assessment of crayfish behavioural and health vital parameters.

1.2. CRUCIAL FRESHWATER SPECIES

Crayfish are characterized by differing among species susceptibility to ambient conditions, therefore occurrence of certain crayfish may be indicative of either good or bad water state. Depledge and Galloway (2005) precisely say that healthy animals are to inhabit healthy ecosystems. Applying this axiom to crayfish, the key-word would be "inhabit". As reported by Svobodová et al. (2012), there is a significant connection between crayfish presence and stream water quality conditions. This also might be rephrased vice versa: if characteristics of some water body are known, one may predict whether crayfish are present there. As an example, the probability of the presence of noble crayfish (*Astacus astacus*) declines with the increase of water pollution (Svobodová et al., 2012). Thus, evaluating the water body on the presence and structure of crayfish community (as well as on other indicative aquatic communities) is the basis of independent biomonitoring approach.

Crayfish are essential ("pivotal") players of freshwater ecosystem (Reynolds and Souty-Grosset, 2012). However, being "just a part of" might be not enough to play such a significant role in biological monitoring, which implies certain qualitatively-numerical verification. Thus, it would be careless to generalise all crayfish as equally good bioindicators. Svobodová et al. (2012) noted only minor differences in water quality for the noble (*A. astacus*) and stone (*Austropotamobius torrentium*) crayfish, while there were observed significant differences in water quality between locations with native crayfish and those inhabited by the invasive spiny-cheek (*Orconectes limosus*) crayfish. In spite of that, such differentiation is only relative, since crayfish *A. astacus* and *A. torrentium* (as well as *A. pallipes* and *A. italicus*) may occur and even prosper in waters with high nitrogen concentrations (Benítez-Mora et al., 2014), increased ammonium (Füreder and Reynolds, 2003; Grandjean et al., 2003; Vlach et al., 2012) and decreased dissolved oxygen levels (Demers et al., 2006).

Many authors agreed that in terms of demands to environmental conditions, even indigenous crayfish could be suggested rather flagships than bioindicators (Füreder and Reynolds, 2003). Anyway, both native and non-native crayfish can be engaged into other biomonitoring approaches, where they would serve as biomonitors of biotic and aquatic statuses. Talking about crayfish as bioindicators, the most often the native species are implied. Generally, invasive species have got well adapted to European waters for many years (Holdich et al., 2009), therefore they can be hardly considered as indicators of the quality of aquatic environment. Nevertheless, these species are successfully used in studies aimed on evaluation of bioaccumulation level of harmful substances in tissues and organs, on toxicological investigation of the whole organism tolerance limits, as well as on elucidation ethophysiological aspects of crayfish as representatives of higher aquatic invertebrates.

1.3. BIOACCUMULATION OF HARMFUL SUBSTANCES IN TISSUES

Aquatic organisms, with no exception for crayfish, are known to accumulate over time certain substances and elements present in water in their tissues and organs. Amount of accumulated element is not just an indicator of what is present in water and what is dangerous for aquatic organisms, but may be a problem if organisms are actively consumed by humans (Aksu et al., 2014). Due to being source of human food, accumulation of toxins in crayfish may be also of a big interest (Wood et al., 2012). Although crayfish are known to accumulate toxins produced by cyanobacteria, as well as toxins containing in eggs and larvae of other aquatic animals, they reported to be very tolerant to toxins impact (Wilson and Williams, 2014), while meat quality of exposed crayfish is not affected by toxins as well (Tricarico et al., 2008). There are various compounds which have been found accumulated in crayfish: polycyclic aromatic hydrocarbons, organochlorides, organochlorine pesticides, polychlorinated biphenyls, residues of organophosphates, pyrethroid compounds and heavy metals (Santerre et al., 2000; Schilderman et al., 1999). Moreover, some metals (such as mercury) are not simply bioaccumulated, but biomagnified in the crayfish (Mason et al., 2000). As far as crayfish occupy intermediate link of the food chain, being facultative omnivorous or carnivorous (Momot, 1995), it should be kept in mind that crayfish can feed on weak or freshly died predatory fish, which is the top-level of mercury biomagnification chain (Subotić et al., 2013). Furthermore this effect is to be observed in crayfish abdominal muscle tissue, while most elements, mainly trace metals residues, are tending to accumulate in the digestive organ, hepatopancreas (Guner, 2007), while aluminium, due to peculiarities of the structure, tends to accumulate ("precipitate") in gills (Walton et al., 2010). Therefore, crayfish are of special interest for investigation of bioaccumulation of harmful substances in their tissues.

1.3.1. Using in toxicological studies

In course of numerous adaptations to varying environments, crayfish can now survive under the wide range of environmental conditions. Due to this, crayfish are widely used as model organisms in toxicological experiments for investigation limits of tolerance and other bio-chemical effects.

Some chemicals may cause not just serious harm to crayfish, but may be applied against other toxic substances as well (Kozák et al., 2009, 2011). Application of crayfish in the toxicological tests can contribute to such allied fields as aquaculture and agriculture. Organochlorine (Santerre et al., 2010) and organophosphate (Buřič et al., 2013; Santerre et al., 2010) pesticides, herbicides (Stará et al., 2014), insecticides (Barbee et al., 2010; Benli, 2014), as well as often occurred nitrates (Benítez-Mora et al., 2014) and heavy metals (Wigginton and Birge, 2007) were examined on the range of crayfish, to demonstrate their frequent tolerance and establish survival thresholds. In particular, such crayfish application found its place in astaciculture, where widely used for disinfection peracetic acid was suggested to be potentially useful for limiting of spread of crayfish plague carrying by non-indigenous crayfish (Kouba et al., 2012). Precisely because of ability to carry crayfish plague, the reason of indigenous crayfish high mortalities, in sum with other detrimental "habits" (high migration ability, reproductive plasticity, competition for food and hiding places, by Holdich et al., 2009 and Jackson et al., 2014), significant body of the literature is devoted to searching of more effective substances for eradication of invasive crayfish from the water bodies (Cecchinelli et al., 2012; Sandodden and Johnsen, 2010).

In spite of natural and anthropogenic "arrangements", most of indigenous species managed to adapt to hardly inhabitable ambient conditions, but when adaptability threshold is exceeded organisms usually die (Gadzała-Kopciuch, et al., 2004). It is very valuable to investigate such threshold, since it may help to prevent the mortality of threatened species, as well as it may help to "accelerate" depopulation of crayfish-invaders.

1.4. ETHOPHYSIOLOGICAL RESPONSES IN WATER QUALITY EVALUATION

Despite that biomonitoring provides quite complex information about environment, there is need to assess status of the single organism, which is possible due to evaluation of its internal function(-s). Among all crayfish vital functions, only respiratory and cardiac activities can be evaluated directly and immediately. Depledge and Galloway (2005) stated that the heart and ventilatory rates within normal range indicate undisturbed organism state. Crayfish have a relatively simple (compare to vertebrates, i.e. fish) organization of the cardiovascular system, although crayfish heart is not just a simple pump, but a complex organ displaying environmental effects through the cardiac alterations (Reiber and McMahon, 1998). Bierbower and Cooper (2010) demonstrated that crayfish reaction to external stressor is quite complex chain of sub-responses, therefore imbalance of one unit may cause dysfunction of the whole organism (Shuranova and Burmistrov, 2010). Cardiac and ventilatory crayfish activities can be measured directly, in "real-time", using either electrocardiography technique (Bierbower and Cooper, 2009) or photoplethysmography (Burnett et al., 2013). This is unique opportunity to monitor such vital functions as heartbeat and respiration, and at once not just avoid traumatizing the animals, but also involve them into such critical process as control for the quality of environment.

Ethological observations in biomonitoring are essential, since behaviour demonstrates the tight relation between environmental impact and physiological state of the animal. Crayfish behaviour is usually observed directly or through the video registration to draw an appropriate ethograms (Bierbower et al., 2013) or to mark out active and motionless periods (Celi et al., 2013).

Burmistrov and Shuranova (1996) found resting locomotor activity to represent the functional crayfish state and reactions to different changes in the environment: the majority of crayfish normally stay immobile most of the day time and are supposed to be active during nocturnal period, having pronounced circadian rhythmicity (Udalova et al., 2012). One of the primary ethophysiological manifestations of crayfish disturbance caused by environmental stressors is disruption of the cardiac circadian cycle. Whereas stabilization of the typical periodically changing physiological functions (locomotor activity, heartbeat, respiration) indicates crayfish adaptation to the environment (Bojsen et al., 1998).

Bojsen et al. (1998) combined cardiac and locomotor activity monitoring and found them tightly related, as well as related with ambient temperature and light intensity. While Bini and Chelazzi (2006) have shown the heart and ventilatory rate to be correlated and decelerate under toxic impact of copper. Crayfish heart rate, locomotor activity and oxygen consumption were shown to be circadian, with significant increase of all parameters during night period, by Styrishave et al. (2007). The background locomotor activity was studied in relation to scaphognathite activity, and revealed that even physically stationary crayfish are wakeful and continuously monitor their environment (Shuranova and Burmistrov, 2010). Finally, the behavioural manifestations were investigated in relation to environmental changes and at once combined with cardiac activity registration by Bierbower et al. (2013). In sum, investigation of more than one characteristics of crayfish state (look into the combination and interaction of several parameters) is critical.

1.5 AIMS OF THE THESIS

The main aims of this thesis were to:

examine suitability of crayfish as bioindicators in course of field and laboratory investigations,

develop and set up the multipurpose biomonitoring system for registration and analysis of crayfish ethological and physiological characteristics,

examine various stimuli (from natural odours to chemical agents) on crayfish and outline the possible pathways of their ethophysiological responses, which would serve the background for further related investigations,

work out and unify the methodology to carry out appropriate biomonitoring studies.

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CHAPTER 2

INVESTIGATION OF CRAYFISH AS WATER QUALITY POSSIBLE BIOINDICATOR

2.1. Kuklina, I., Kouba, A., Kozák, P., 2013. Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review. Environmental Monitoring and Assessment 185, 5043–5053.

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Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review

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Abstract Water quality monitoring using fish and crayfish as bio-indicators requires an understanding of the state of pollution of waters, choice of bio-indicators, physiological and behavioral endpoints of fish and crayfish, and principles of the methodology and their potential applications. Here, we discuss telemetry, acoustic monitoring, vision-based monitoring, measures of ventilatory activity, electrocardiography, and fiber-optic plethysmography. Assessment of water quality must be based, not only on physicochemical characteristics of the current environment as determined by chemical analyses, but also on observations of the physiology and behavior of its inhabitants. Real-time biomonitoring is suggested as the most reliable method, since it incorporates living organisms into the system to serve as biosensors. The potential application of the methods discussed includes use at water treatment plants and water supply stations for prevention of hazardous toxicological events, and, for aquaculture, in ponds, lakes, and aquariums for monitoring growth, population size, and behavior traits.

Introduction

Maintenance of high-quality natural aquatic systems is important, as contaminated water is a source of disease in humans and other organisms (Serra-Toro et al. 2010). Water quality management must be based on the assessment of harmful impacts on the aquatic environment and their effective control and minimization (Bowen and Depledge 2006). Traditional toxicity tests and chemical-specific sensors cannot provide comprehensive real-time information relating to toxic events in an aquatic system; aquatic organisms must be included as a major component of assessment processes (van der Schalie et al. 2001). Reports of water quality monitoring by analysis of the physiological and ethological responses of native hydrobionts have shown that the most suitable organisms for this purpose are fish (Shedd et al. 2001) and crayfish (Fedotov et al. 2002). Biomonitoring is the most appropriate method for environmental study and for control of both industrial and natural water quality by virtue of its inclusion of living organisms into automated water monitoring systems as biosensors of the physicochemical and biological characteristics of water (Kholodkevich et al. 2008). Biomonitoring often includes collecting the organisms from representative sites and referring their quality level based on proportions of crayfish

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species present (Reynolds and Souty-Grosset 2012), as well as sampling the tissues from collected organisms for evaluation of respective pollutants bioaccumulation level (Alcorlo et al. 2006). Two of the most significant challenges in biomonitoring are quantification of the relationship between anthropogenic disturbance and chemical and biological indicators across wide geographic areas and the development of appropriate tools to assess ecological status (Archaimbault et al. 2010).

Organisms used as bio-indicators must have higher sensitivity than the best chemical indicators (Gadzała-Kopciuch et al. 2004). In aquatic animals, behavioral and physiological (cardiac and respiratory rates) changes serve as indicators of sensitivity to contamination, since the organism will present as healthy if it lives in a suitable environment (Depledge and Galloway 2005). Parks et al. (1991) notes some fish and crayfish species worldwide distribution throughout the water bodies is to be indicator of appropriate water quality. While Roussel et al. (1999) adds that cool temperate Salmonidae are commonly regarded as bio-indicators of high water quality in the northern hemisphere. Due to sensitivity to environmental pollution, indigenous freshwater crayfish are also considered valid bio-indicators of good waters, even despite some exceptions which have been reported (Füreder and Reynolds 2004; Reynolds and Souty-Grosset 2012).

Aquatic pollutants

There is a strong relationship between human activity and disturbance of the aquatic environment (Hodkinson and Jackson 2005). Aquatic animals can detect, discriminate, and respond to a variety of chemicals (Derby and Sorensen 2008) and respond sensitively to both beneficial and harmful chemicals. Causes of deteriorating water conditions are acidification and the presence of nitrogen and phosphorus, heavy metals, organic toxicants, and pesticides (Bloxham et al. 1999).

Some of the most widespread water pollutants are nitrites. Nitrite exposure to fish can affect ion regulation and respiratory, cardiovascular, endocrine, and excretory processes, as well as fish behavior (Kroupová et al. 2005). High or long-term nitrite exposure may reduce the survival and fitness of freshwater invertebrate populations (Harris and Coley 1991) including crayfish (Yildiz and Benli 2004). It has been reported that nitrite and chloride are reciprocal inhibitors of uptake (Cheng and Chen 1998). Thus, chloride (as well as bromide) can

inhibit nitrite toxicity. This is applicable to aquaculture, due to chloride's ability to reduce the negative impact of high nitrite concentrations on crayfish (Kozák et al. 2005, 2009).

Water disinfection presents a potential problem for aquatic life. Protecting users of water, as well as its inhabitants, from disease caused by waterborne pathogens is essential (Hua and Reckhow 2007) but may result in the formation of harmful byproducts. Chlorination and chloramination, with and without ozonation, are used for both drinking water disinfection (Moore and Calabrese 1980; Hua and Reckhow 2007) and in aquaculture (Speare et al. 1996; Powell and Perry 1999). Chloramine degrades in water, releasing free chlorine which acts as an irritant and induces acute respiratory problems in fish and acid–base disturbances in fish blood (Powell and Perry 1999).

Some water contaminants, such as heavy metals, are slow to bioaccumulate and do not immediately appear in crayfish (Styrishave et al. 1995; Styrishave and Depledge 1996; Bamber and Depledge 1997; Kouba et al. 2010). Heavy metals may be transformed into persistent metallic compounds with high toxicity that bioaccumulate in fish (Zhou et al. 2008). Moreover, some persistent substances such as DDT, polychlorinated biphenyls, mercury, and selenium can accumulate in fish and present a big threat. Heavy metals are accumulated through food chains to toxic concentrations and can represent a potential threat to the health of animals and humans.

Other pollutants have equally harmful effects on fish and crayfish, including cardiac and respiratory distress, loss of locomotor ability, and unusual behaviors (Handy and Depledge 1999).

Bio-indicators

The selection of bio-indicator for an investigation is critical. A bio-indicator is an organism that readily reflects the state of the environment and represents the impact of environmental change on a habitat (McGeoch 1998 in Hodkinson and Jackson 2005; McGeoch et al. 2002). A variety of organisms can be used in biomonitoring, but fish and benthic invertebrates are the most common, and each of these has advantages and disadvantages (Resh 2008). Ideally, test organisms (bio-indicators) should have the following characteristics: (1) having high ecological relevance; (2) susceptibility to stressors in the field and

in the laboratory; (3) having wide geographic distribution; (4) being easy to culture and maintain under laboratory conditions; (5) having a relatively high reproductive rate; and (6) with the ability to yield reproducible data under controlled laboratory conditions (Kane et al. 2005).

Fish were the sentinel organisms originally selected for biological monitoring systems, and they continue to be a popular choice (Shedd et al. 2001), as it is relatively easy to determine their numbers, biological diversity, and behaviors (Gadzała-Kopciuch et al. 2004). Fish can be effectively used in research due to their constant direct contact with the aquatic environment, ecological relevance in many natural systems, ease of culture, and ability to be induced into reproductive readiness (Kane et al. 2005).

Reports on bio-testing of waters through the use of physiological and ethological responses of hydrobionts have also suggested testaceous invertebrates such as mollusks, crabs, and crayfish as potential test organisms for assessment of water toxicity (Fedotov et al. 2002).

The crayfish is an important bio-indicator species, being physiologically and behaviorally adaptable to environmental changes, while also being sensitive and susceptible to stressors. Crayfish are known to exhibit a wide range of rapidly changing behaviors as well as the ability to assess and respond rapidly to environmental stimuli (Bierbower and Cooper 2009). Ecological relevance, an external hard shell, and susceptibility to water contamination make the crayfish attractive as a bio-indicative organism (Kholodkevich et al. 2008).

Behavioral and physiological responses

Two basic characteristics are used to identify the responses of an observable animal to alterations in the environment, changes in behavior, and changes in cardiac and respiratory rates.

Why study behavior?

Behavior of living organisms is directly related to the state of the environment, reflecting a unique relationship of the organism to its environment, relative to its physiology and ecology (Little and Brewer 2001). Since behavior serves as a link between physiological and ecological processes (Scott and Sloman 2004), it is defined as a complex sequence of quantifiable actions, operating through the central and peripheral nervous systems, and an integrated reflection of processes essential to life, such as feeding, reproduction, and predator avoidance (Kane et al. 2005). Thus, behavior is a structured integration of behavioral endpoints (movement, velocity, path changing, space utilization, distance from center, etc.), and it serves as a valuable tool to discern and evaluate effects of exposure to environmental stressors (Kane et al. 2004, 2005).

Fish

The study of fish behavior is being applied to water quality monitoring and toxicity identification (Chew et al. 2009). The rapid behavioral responses seen in fish make them ideal subjects for observation, and analysis of fish behavior has been a popular approach to detect changes in the aquatic environment. When the environment is negatively affected, fish display variations in behavior such as avoidance reactions and changes in swimming ability. The schooling of fish is one of the most familiar forms of animal social behavior (Partridge 1982), and schooling is one of the typical endpoints that reflects, not only hierarchical relationships inside the school, but describes behavioral trends related to alterations in the environment. Change in their environment may be first indicated by avoidance of an area by a school, before the behavior reaches the level of the individual fish. Each rapid change in the distribution of fish, first reflected in schooling, is evidence of environmental change. Behavior alterations can be measured as endpoints for sublethal toxicity and serve as tools for environmental risk assessment and analysis of toxicological impact (Kane et al. 2004).

Crayfish

The relative simplicity of the nervous system compared with vertebrates, and the ease with which they can be used for experimentation, make crustacean models useful for study of mechanisms underlying behavior (Li et al. 2000).

Crayfish also continually alter their behavior according to stimuli. Locomotor activity of crayfish has been shown to increase markedly following exposure to certain chemical stimuli (Hebel et al. 1997). Typical behavior alterations in crayfish are avoidance of areas of contamination or over-illumination, elevating of chelae, dramatic escape reactions, initiating of fighting with conspecifics (Schapker et al. 2002), and

movement of flagellar exopods (Burmistrov and Shuranova 2010). This latter reaction has several functions: creation of water currents for purposes such as sending and receiving chemical signals, preventing ingestion of small particles, and detection of water currents produced by the scaphognathites (Burmistrov and Shuranova 2010; Breithaupt 2001). Burmistrov and Shuranova (2010) observed flagellar movements in (1) crayfish trained to receive food after illumination of the tank. They showed flagellar movements immediately following illumination, lifting the anterior part of their body, which made their mouthparts visible; (2) crayfish moving rapidly to avoid illuminated areas; and (3) female crayfish placed in a tank that contained a male and vice versa. Thus, flagellar exopods may serve as tools to express the internal state of crayfish (Burmistrov and Shuranova 2010), which may reflect the state of the aquatic environment.

Why study heart end ventilatory rates?

Heartbeat and respiration within the normal range are indicators of the normal state of an organism (Depledge and Galloway 2005). The cardiovascular system of an animal must provide adequate perfusion of all tissues for the purposes of supplying O_2, nutrients, and hormones and for removal of wastes. Its performance must be adaptable to meet the changing needs of its "host" (Wilkens 1999). An animal's heart rate can signal the combined impact of natural and anthropogenic stressors on the organism and may impart information about the availability of energy sufficient to allow the organism to grow, feed, and reproduce normally (Depledge and Galloway 2005). Imbalance in the cardiac or respiratory systems may indicate substandard environmental quality.

In biomonitoring, fish are mainly used in observations of behavior alterations associated with water contamination (Scott and Sloman 2004). A large body of scientific work is also devoted to the study of fish cardiac and respiratory systems. For example, electromyogram activity recorded in fish axial musculature is proportional to locomotor activity and can be correlated with oxygen consumption, helping to give a complete picture of metabolic rate (Cooke et al. 2000). Metabolic rate within the normal range indicates a normal environmental state. Ventilatory parameters known to be sensitive to toxicity include ventilation rate and depth, cough, and gill purge rate (van der Schalie et al. 2001).

It is commonly suggested that only vertebrates have a sufficiently developed circulatory system to be reliable bio-indicators, but it has been shown that the crustacean heart is not a simple pump but a complex organ that responds to specific physiological needs (Reiber and McMahon 1998). Crayfish are extremely sensitive to changes in water, especially to pollutants. Changing parameters of heart and ventilatory rates display reactions of crayfish to stimuli. Even when crayfish remain stationary, heart and ventilatory rates can vary greatly. Physiological observations provide the most precise assessment of the status of water. Heartbeat, ventilatory activity (respiratory rate and movement of scaphognathites), and flagellar exopod activity can be monitored for the evaluation of the crayfish state (Shuranova and Burmistrov 2010). Consequently, heart and ventilatory rates serve as reliable tools for evaluation of animal health and may have implications for assessment of the aquatic environment (Bierbower and Cooper 2009).

Methods for monitoring the aquatic environment

Several methods are commonly used for observations of the external and internal states of aquatic organisms and evaluation of the aquatic environment.

Acoustic monitoring

Up to now, there are reports in literature about an acoustic approach dealing with fish only. Acoustic monitoring is a technique using sound waves to remotely measure population density, behavior, and growth rate of fish and to detect unexpected behaviors. Acoustic monitoring utilizes a strong acoustic reverberation in a tank to obtain an accurate measure of sound scattering from the fish (Conti et al. 2006). A pulse is transmitted in the tank using a single emitter, while a time series composed by the echoes of the reverberations in the tank are recorded on one or more receivers simultaneously. The recorded echoes, scattered by the swimming fish, can be used to measure integral acoustic intensity, which is related to the number and size of fish as well as to their behavior (Conti et al. 2006).

Acoustic telemetry (biotelemetry) is also used for the remote measurement of biologically relevant data, including behavioral, physiological, and environmental data. This method enables researchers to document, for long uninterrupted periods, how undisturbed

organisms interact with one another and their environment in real-time (Cooke et al. 2004). Data are transmitted to the researcher through the use of an electronic tag, i.e., an ultrasonic transmitter which is attached, internally or externally, to the fish. Acoustic telemetry is used in brackish and saline water and in deep freshwater lakes and reservoirs (Goetz 2006) to analyze swimming behavior variables such as trajectory complexity, spatial distribution, and motion (Anras and Lagardère 2004).

Fish heart rate (electrocardiogram and electromyogram) acoustic telemetry (Armstrong et al. 1989) is used to obtain in-depth information about fish, and, thus, about the aquatic environment. Recording is similar to biotelemetry, but the electrodes detect electropotentials within the axial red muscle, and the pulse emitted from the transmitter provides information on integrated electrical activity—a direct electromyogram (Cooke et al. 2000).

Sonars and lidars allow accurate remote identification and monitoring of fish distance and motion using underwater sound propagation and laser impulses, respectively (Steig and Johnston 1996; Churnside et al. 2003).

Vision-based real-time monitoring

From 1980 to the early 1990s, the traditional shadows and stereophotographic methods or a single video camera with mirrors (Pereira and Oliveira 1994) were used for vision-based real-time monitoring.

Current vision-based methods automatically monitor the behavior of fish schools with real-time images to gather information about the aquatic environment. Vision-based real-time monitoring allows: (1) monitoring of a school as opposed to an individual fish; (2) observation of fish without introducing stress; (3) analysis of behavior traits of fish schools; (4) immediate detection of variations in water conditions (Chew et al. 2009).

Real-time images are captured using a camera fixed above an aquarium. Using information provided by its physical dimensions and position mapped on the photographic images, each fish is represented by a set of coordinates of the pixels it occupies (Chew et al. 2009). Using vision-based real-time monitoring, one can observe three significant behavioral endpoints to determine the water state: activity level, school distribution, and social interactions within the school (Serra-Toro et al. 2010). The activity level of the fish school can be determined by swimming speed and the complexity of the swimming path. The distribution of a fish school is determined by the population of fish in areas of the tank (Chew et al. 2009).

Additionally, Tuthill and Johnsen (2006) reported the use of polarized light to be good in biological monitoring when video-tracking crayfish behavior. Although the ecological role of this solution remains undefined for crayfish, evident use was revealed for study of escape responses which are of big interest and for biological monitoring particularly.

EthoVision

EthoVision is an important technique designed to measure behavioral (ethological) alterations such as activity, movement, and interactions of animals. It is an integrated system, comprising software and hardware components. A charge-coupled device video camera records the area containing the subject animals. The analog video signal is digitized by a frame grabber and fed into a computer. The frame grabber can either digitize the camera's signal directly or take input from a video cassette recorder. The software analyzes each frame to distinguish the tracked objects from the background on the basis of either their grayscale (brightness) or their hue and saturation (color) values. Having detected the objects, the software extracts the required features, for example, the position of the mathematical center of each object (center of gravity). The software is able to analyze the movement of each animal (Noldus et al. 2001).

Video multitracking in fish

The 2D multitracking system is able to track up to 100 unmarked individuals in a single arena. This system extrapolates a trajectory of each individual (for example, fish) and analyzes recorded sequences that are several minutes long. A mirror placed at a 45° angle reflecting the bottom of an aquarium provides the potential to observe fish movements from several directions for more complete behavior analysis (Delcourt et al. 2006, 2009).

Video recording of scaphognathite movement in crayfish

Shuranova et al. (2003) described a procedure for video recording scaphognathites (SG) and mouthpart appendage movement. A crayfish was fastened on its side in a glass vessel with the bottom covered by a

layer of transparent material. An emitter of infra-red light was situated under the vessel, and a small photo-transistor was placed above the area of the crayfish prebranchial chamber. Thus, optical signals and electrical activity near the prebranchial chamber were recorded in complete darkness.

Video-based monitoring is aimed at behavioral studies and also allows monitoring of crayfish SG movement, from which it is possible to judge the state of crayfish and its environment, as is shown by Burmistrov and Shuranova (2010). At the same time, crayfish acoustic signaling was video-recorded by Favaro et al. 2011. In order to identify the movements of the appendages involved in sound production, the crayfish was video-taped, focusing on the aperture of the branchial chamber. Despite that the study was aimed for revealing the mechanism of sound production, this procedure can be used in biomonitoring purposes as well.

Real-time biomonitoring

In practice, biomonitoring is increasingly used for control of water quality by inclusion of living organisms as sensors of the physicochemical and biological characteristics of water in automatically monitored systems. Existing physicochemical analyses may require considerable time and provide information on only a limited list of water pollutants. Unlike traditional methods, real-time biological monitoring provides insight into water conditions in general and can do it immediately (Kholodkevich et al. 2008) to prevent polluted water from entering a municipal water system.

The use of aboriginal animals as indicators in biomonitoring is an interesting and important facet of the qualitative study of the aquatic environment. Biomonitoring utilizes measurements of heart and ventilatory rates, which provide a direct measure of the internal state of animal (Bierbower and Cooper 2009).

Fish ventilatory detection system for real-time toxicity evaluation

The following method is aimed at long-term automated biomonitoring of water quality based on ventilatory activity and fish movement patterns and is applied at water treatment facilities for real-time control of the effluent prior to discharge. The ventilatory signals from individual fish placed in a chamber are monitored by an electrode suspended above and below. The

electrical signals are amplified, filtered, and fed into a computer for analysis (Shedd et al. 2001). The parameters measured are ventilatory rate, ventilatory depth, gill cough frequency, and whole body movement. The system monitors control fish simultaneously with fish exposed to effluent for evaluation of atypical or adverse responses (van der Schalie et al. 2001).

Invasive biomonitoring in crayfish and other crustaceans

Invasive biomonitoring implies direct insertion or implantation of measuring equipment (wires, sensors, or electrodes) to connect an animal to a measuring device.

Bierbower and Cooper (2009) described a method of invasive biomonitoring based on electrocardiography that included crayfish as a part of the measuring circuit. Two isolated wires were placed under the dorsal carapace directly over the heart to record heart rate. These two wires were positioned to span the heart in a rostral–caudal arrangement to ensure an impedance measure during each contraction. A second pair of wires was placed under the cuticle in the rostral area of the gill chamber to monitor the ventilatory rate. Wires were inserted through holes drilled in the carapace and cemented in place with cyanoacrylate ester and accelerator. Heart and ventilatory rates were observed as differences of dynamic resistance (i.e., instantaneous voltage emergent between applied electrodes). Thus, it provided both an electrocardiogram (Li et al. 2000; Schapker et al. 2002) and an electromyogram (EMG) (Listerman et al. 2000).

Chikamoto et al. (2008) obtained EMGs of the four muscles of thoracic legs of crayfish and simultaneously video-recorded leg movements to quantitatively characterize walking behavior either chemically initiated or spontaneous. Extracellular recording from one of the walking leg muscles was accomplished with a pair of silver wires inserted into the relatively immobile region of the muscle through fine holes drilled in the cuticle. The holes were sealed and the wires glued to the cuticle. Thus, electrical signals from activated and non-activated crayfish muscle were obtained.

Sigel (1998) measured cardiac output, heart rate, and ventilatory frequency of the marine crab. Flow within two arterial systems (anterolateral and anterior) was assessed using a pulsed Doppler flowmeter to detect changes in regional blood flow and vascular resistance. Blood flow in arteries was detected, and the frequency of the pulsation (reflected waves) was proportional to

the velocity of the blood flow (Sigel 1998). Ventilation frequency was measured using pressure transducers connected to the epibranchial chambers. Ventilation frequency was determined by counting the peaks in the ventilation pressure waveform, which correspond to scaphognathite movement. Cardiac output was calculated as the sum of all arterial flow. Heart rate was determined by counting the peaks in the flow records, and stroke volume was calculated by dividing cardiac output by heart rate (De Wachter and McMahon 1996).

Similar measurements of hemolymph flow and branchial and hemolymph pressure have been conducted on crayfish and lobsters. Arterial hemolymph flow has been measured using a directional ultrasound pulsed Doppler flowmeter. Ventilation and heart beat frequencies were also measured in both species under hypoxic conditions (Reiber and McMahon 1998). Hypoxia caused by excessive contamination is one of the environmental factors affecting immune responses of aquatic animals (Le Moullac and Haffner 2000).

Non-invasive biomonitoring

The majority of current scientific literature is devoted to the non-invasive study of living organisms.

The computer-aided physiological monitoring system was a first step in this method and is still in use. Initially, it was a system using infra-red sensors attached to the shell of an animal to monitor heart rate (Aagaard et al. 1991). The optoelectronic technique has also been used to record ventilatory activity (periodic movement of the scaphognathite muscle) of crayfish (Bini and Chelazzi 2006).

Modified non-invasive biomonitoring is based on plethysmography (PG), a method for study of vascular tone and blood flow in thin vessels. Initially, PG was used in space medicine. It is based on a graphic recording of pulse and fluctuations associated with blood-filling of vessels (Gil et al. 2006).

Photoplethysmography (PPG) is an optical measurement technique that can be used to detect blood volume changes in microvascular tissue. The basic form of PPG requires only a light source to illuminate the tissue and a photodetector to measure variations in light intensity associated with changes in perfusion reflected in the catchment volume (Allen 2007).

Fiber-optic method can be used to record and analyze cardiac activity of crayfish, crabs, and mollusks, since such animals possess a hard shell for sensor attachment (Kholodkevich et al. 2009). An infra-red light beam formed in the laser fiber-optic photoplethysmograph is transmitted to the animal by a thin optical fiber, and a small sensor, connected with optical fiber, attached to the carapace illuminates the heart area with scattered light. The optical signal is modulated by heart muscle contraction. After appropriate amplification and filtration, the analog signal is converted to digital form and transmitted to a computer (Fedotov et al. 2000, 2006). The resulting photoplethysmogram can be further analyzed by mathematical and statistical methods (Fedotov et al. 2009).

Non-invasive recording of electrical field potentials

Shuranova et al. (2003) used a non-invasive technique for recording bioelectric potentials produced by SG movement in crayfish. An electrical potential was recorded at points near the prebranchial chamber and inside it, using one or two recording electrodes fixed to the carapace with plasticine. Electrical potentials at the anterior opening of the prebranchial chamber and in other appendages located near the mouth opening were recorded using the same method.

Using other aquatic organisms for water quality monitoring

Other aquatic organisms have been reported as appropriate bio-indicators for water quality monitoring. Mollusks are widely used since they have several important advantages (Salánki et al. 2003), among which are ease of handling, and, most importantly, rapid response to changes in the aquatic environment (e.g., temperature) (Borcherding 2006). Two biological signals, the percentage of open mussels and the number of valve movements, can be used to indicate toxic water conditions (Oehlmann and Schulte-Oehlmann 2002). Recently, change in mollusk cardiac rhythm has also been used as an indicator of aquatic environment alteration (Curtis et al. 2000; Kholodkevich et al. 2009). Moroishi et al. (2009) used a freshwater bivalve as a bio-indicator for a non-invasive technique based on changes in the external magnetic field measured by a magnetic device called a Hall element. The valve movements of the bivalve were continuously recorded as output voltage from the Hall element sensor.

Generally, only aboriginal animals are sufficiently adapted to peculiarities of their surroundings and its changes to make them suitable as test species, and marine lobsters (Rose et al. 1998), mollusks (Moroishi et al. 2009; Santini et al. 1999), crabs (Bamber and Depledge 1997; Brown et al. 2004), benthic macroinvertebrates (Ren et al. 2007), and aquatic snakes (Campbell and Campbell 2001) are also being used as bio-indictors in specific areas. Similar to crayfish, non-invasive optoelectronic technique was tested on crabs (Depledge and Lundebye 1996; De Pirro et al. 1999), tropical scallops (Brown et al. 2004; Santini et al. 1999), crayfish (Bini and Chelazzi 2006), and amphipods (Calosi et al. 2003) giving successful results.

Conclusions

A wide variety of methods for water quality monitoring are available to identify a great variety of water pollutants in the aquatic environment, as well as wide variety of species that can be used as bio-indicators. Fish and crayfish are considered the most interesting models, since they serve as indicators of introduced contaminants through both behavioral and physiologic reactions reflecting environmental conditions. Physiological assessment (cardiac and respiratory activity) in combination with behavior observations (feeding, locomotion, inactivity, social interactions) give a complete understanding of environmental impact on the organisms (Bierbower 2010) serving as the water quality monitors.

The advantage of the described monitoring methods is that common fish and crayfish can serve as real-time monitors in bodies of water such as rivers, coastal waters, and ponds, as well as water treatment facilities, water supply stations, and artificial reservoirs. Under laboratory test conditions, in the absence of environmentally realistic challenges, it is difficult to accurately determine the potential impact of contaminants on animals in natural conditions (Bamber and Depledge 1997). Learning to adapt the described methods for use in the monitoring of conditions in the natural environment is an important task. Without preliminary laboratory tests, these methods cannot be used on a large scale, such as in water treatment plants or water supply stations. Only under laboratory conditions is it possible to directly test animal reactions to specific compounds, to make it possible to translate these reactions to animals in the natural environment.

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CRAYFISH AS TOOLS OF WATER QUALITY MONITORING

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1. INTRODUCTION

While numerous authors are working hard at attracting public attention to crayfish protection, management and conservation (a very precious intent), authors of this chapter would like to stress the importance of crayfish in protecting human wellbeing. In fact, crayfish are known to be characteristic representatives among the keystone species in freshwater ecosystems (Reynolds and Souty-Grosset, 2012), which enables us to rely on crayfish indices while estimating the ambient status of their natural habitats and those carefully simulated in labs. The latter is done to imitate various water conditions, thus screening and predicting crayfish reactions to changes in those specific conditions, should they happen in nature. At first, it may sound irrational to move crayfish from their natural homes to simulated "close-to-natural" laboratory conditions, but by exposing them to stress, their reactions can be tested. The elucidative point is that the ethophysiological responses in crayfish, studied during stress, are functions of their environmental adaptation (Burmistrov and Shuranova, 1996). For us, it means an opportunity to observe, study and forecast, as far into the future as possible, how they would behave on ethological and physiological levels in common environments if certain conditions, pretested in vitro, occurred.

Living in a scientifically progressive century, where nearly all significant discoveries and phenomena have been made and elucidated, there is almost no chance to surprise the public by something principally new. Nevertheless, there is unquestionably a capacity to use proven knowledge in new and practical ways. The crayfish have been irrefutably shown to be excellent water quality bioindicators (Füreder and Reynolds, 2003; Reynolds and Souty-Grosset, 2012). We know their ethophysiological indices tend to vary with the ambient conditions (Bloxham et al., 1999; Lowe et al., 2010). So why would we not apply this knowledge in a beneficial way? The biomonitoring actually does perform that.

This chapter is presented as an extended summary of the biomonitoring approaches that are already in practice or will eventually be applied, with a particular accent on the potential of continuous real-time experimentation. There have been numerous studies presented in the scientific arena that prove it is possibile to measure certain crayfish parameters which are related to the status of the aquatic environment. Many approaches were shown to give instantaneous ("real-time") measurements, but we believe a large part of them can be developed into continuous ("long-time") monitoring techniques.

2. CRAYFISH SELECTION

Depledge and Galloway (2005) coined a significant saying: "healthy animals, healthy ecosystems." The animal cannot be fully healthy if living in an ecosystem that is not fully healthy. Likewise, an ecosystem cannot be considered complete and healthy if it contains unhealthy inhabitants. These statements can hardly be separated, because nature is always in balance, as are certain formulae for successfully implementing biomonitoring.

Crayfish are extremely useful model organisms because of their many benefits: relatively simple cardiovascular systems make monitoring heart and ventilatory rates easy, sensitive nervous systems make stimulation and observation of reactions easy, uncomplicated reproduction as well as straightforward growing and maintenance in laboratory conditions simplifies acquisition of animals with no additional harm to the natural ecosystem, and hard exoskeletons make experimentation easy. In other words, crayfish possess all the requirements suggested for bioindicator selection (Kane et al., 2005).

Numerous crayfish species from around the world were successfully used in research studies: *Astacus astacus, Astacus leptodactylus, Austropotamobius pallipes, Cherax destructor, Cherax tenuimanus, Euastacus sulcatus, Orconectes australis packardi, Orconectes limosus, Orconectes rusticus, Orconectes virilis, Pacifastacus leniusculus, Procambarus clarkii, Procambarus cubensis* and even *Astacopsis gouldi*. Such variety meets an additional requirement of the "ideal" bioindicator – geographical diversity – which makes possible the use of various species in labs all around the world.

Historically, males appeared to be the "defenders" of some "environment" or other. This can be extended to crayfish, where males are chosen to monitor the aquatic environment, becoming its "protectors." In contrast, females supposedly tend to have higher heart and ventilatory rates, especially under stress conditions (Cooper et al., 2011). Thus, for the majority of experiments, it is more reasonable to use males, because they are less subjected to environmental stress and do not generally undergo the dramatic changes seen in females. Size can be a limiting factor for biomonitoring if the approach implies a need for tags or sensors attached to the animal. A carapace of necessary area is needed to affix such monitoring units. Therefore, proper size is essential not just for manipulation; it is crucial for the animals, because if they are too small, their movements and other vital activities can be restricted by these devices. However, external characteristics are not the only preferences applied when selecting animals. Their physiological indices can and should be considered as well. Circadian cardiac rhythmicity reflects whether day rest and night active states are fulfilled, which is an indicator of crayfish undisturbed state (Udalova et al., 2012). Finally, for long-term study, hemolymph has been shown to be a suitable parameter used when forming a bioindicator group because it reflects the crayfish functional (i.e., health) state (Sladkova and Kholodkevich, 2011).

We will not describe it in detail here, but experimental crayfish obviously should possess very similar external and internal characteristics, and the experimental purpose, duration, conservation status and previous experiences should be taken into account as well. Aside from being water protectors, crayfish are considered aquatic biomonitors and predictors because of particular characteristics that they monitor and expose, as described in the following sections.

3. APPROACHES FOR WATER STATUS ASSESSMENT

Here, we describe the variety of approaches involving crayfish as biosensors of their ambient conditions. We also discuss methods using crayfish and other crustaceans (shrimp, lobster, crab or amphipods) or mollusks (bivalves or gastropods) and even allied arthropods (spiders) as experimental subjects (Coelho and Amaya, 2000) that can potentially be applied for crayfish biological monitoring. To compare various features of existing methods, they should be applied in their chronological sequence, as they appeared in the literature.

3.1. Tracking behaviour

Expected behaviours of crayfish exposed to various environmental conditions are determined with no consideration of feeding or reproductive activities. Overall, animal behaviour demonstrates the strongest relationship between environmental impact and physiological state. It is twice as useful to monitor the peculiarity of their behaviour in various forms (patterns), such as movement vs. motionless, exploration vs. hiding or interaction vs. avoiding.

Behavioural studies have become much less laborious and more purposeful with technological advancements in behavioural measurements (Kruk, 1997).

3.1.1. Camera-based

In use for several decades (Bolt and Naylor, 1985), systems based on video signals remain the most versatile for automated analysis of behavioural data (Kruk, 1997). Biomonitoring based on video camera observations presents a common, reliable approach giving in-depth information on animal behaviour.

Well water and sufficient light are integral factors in normal aquatic life. However, they can cause certain disturbances when video monitoring. In the dark, when most of crayfish are active, the natural light intensity is too low for observation, and waterborne vibrations are too high, also causing complications in the monitoring process (Patullo et al., 2007).

Solving these challenges result in better data quality and wider experimental opportunities for astacologists. The primary solutions for crayfish multipurpose video-monitoring are considered in paragraphs below.

Movement patterns

A large segment of the published research is devoted to studying crayfish behaviour under various stimuli, introduced to crayfish externally (Celi et al., 2013; Schapker et al., 2002) and internally (Panksepp and Huber, 2004). Even when experiments were primarily focused on revealing specific behavioural tendencies or drawing crayfish ethograms (Bergman and Moore, 2003; Bierbower et al., 2013), at some experimental stage, all studies used video recording devices to register, store and analyse ethological data. In some cases, the cameras were placed either above (Horner et al., 2004) or below (Panksepp and Huber, 2004) experimental arenas, and could be either automatically moved to track animal paths (Horner et al., 2004) or permanently fixed throughout the experimental trial (Celi et al., 2013).

To improve recordings in low light, backpacks with two red light-emitting diodes were attached to spiny lobsters (*Panulirus argus*) to facilitate tracking by a camera fixed above the experimental tank (Horner et al., 2004).

Video tracking of crayfish (*Orconectes rusticus*) was enhanced using extra illumination from beneath the experimental arena and diffused light from above it (Panksepp and Huber, 2004).

Tuthill and Johnsen (2006) used polarised (partially linearly) light for effective biological monitoring when video tracking the behaviour of *Procambarus clarkii*. Although a practical ecological role of this solution is not defined, the study revealed that escapes occurred 5 times more frequently under polarised light, a result of great interest for biological monitoring, particularly because escape can signal stress stimuli, such as predators or chemical threats.

Another innovative solution filmed *Cherax destructor* in infrared light in specially constructed recording arenas (Patullo et al., 2007). For more detail, a black-and-white charge-coupled device camera with six built-in near-infrared light-emitting diodes was fixed above the experimental tank while each short end of the tank was equipped with two additional light sources positioned above the walls and angled so as not to point directly perpendicular to the tank bottom, minimising reflection. Such a set-up enabled closer observation of crayfish behaviour in natural situations than other video methods because infrared light facilitates observation in the dark, but resolution is sacrificed.

Burmistrov and Shuranova (1996) found that resting locomotor activity represented the functional crayfish state as did reactions to various changes in the environment. However, intensive use of this observation is limited by very low crayfish motor activity at rest. On the other hand, it can be beneficial, because if crayfish rest most of the time, changes in their resting heart and ventilatory rates are easier to quantify. Of course, it becomes more evident when they begin to move, but some cumulative effects can occur: when either heart or ventilatory rate is measured together with motion and those parameters change simultaneously, an integral stress-rate increase can be observed. Therefore, we will devote more attention to this issue (See Section 3.3.).

Orienting and locomotion traits

It is necessary to study orienting behaviour because it tells not only about topographical abilities, but also avoidance reactions caused by environmental stress. In Basil and Sandeman (2000), the paths of *Cherax destructor* locomotor activity were video-taped and digitised, so that the travelled distance and velocity could be evaluated over time intervals. It would be unwise to neglect such a monitoring opportunity, especially when it can be so easily performed.

To describe locomotor patterns in detail, *Procambarus clarkii* were monitored using a filming device consisting of a wheeled table fitted with a rotating platform carrying a video monitor and a crane, the end of which was affixed a video camera. The crayfish were filmed from above during their homing walks, assuring that they always stayed in the centre of the net-like control screen by moving the table. The camera was directly connected to the video display screen (Jamon and Clarac, 1995).

Interaction

We have mentioned the complication of faint or absent illumination during video observation of crayfish nocturnal activities. Their interactions have been filmed using a video camera illuminated by white lights mounted on an underwater housing, which held the camera (Bergman and Moore, 2003); however light often appeared to be a threat, so resultant interactions had to be excluded during data processing. Although crayfish are primarily active at night, making it difficult to distinguish excited or disturbed states, there is always the possibility of a substandard situation when it is dark.

Flagellar exopods

The behaviour of *Procambarus cubensis* was documented using a video recorder placed near the front wall of individual containers. Movements of the flagellar exopods and scaphognathites were recorded when they became visible through the transparent wall of the prebranchial chamber (Burmistrov and Shuranova, 2010). An interesting feature of the flagellar rhythm is the constancy of its rate. The frequency of flagellar beatings was very similar among individuals (Shuranova and Burmistrov, 2009). This peculiarity might be beneficial in biomonitoring studies, because it allows distinguishing between normal and excited crayfish conditions. Experimental confirmation showed that excited crayfish exhibited significantly higher flagellar exopod activity in heterosexual conspecific presence (Burmistrov and Shuranova, 2010), and a similar reaction might be observed with other disturbances, including those involving chemical agents.

3.1.2. Passive acoustic monitoring

The lack of information on the abilities of crustaceans to exhibit underwater sounds was reiterated by Buscaino et al. (2011). Subsequently, it was shown how the acoustic signals emitted by the red-swamp crayfish *(Procambarus clarkii)* were recorded using a calibrated hydrophone connected to a digital acquisition card, which was calibrated using pure tone sine waves at various frequencies and intensities, produced by a signal generator. The audio system was synchronised with the video system, which consisted of two low-light cameras, one centred above the experimental tank and one under water on the side of the tank (Buscaino et al., 2012).

Regardless of the species used in this approach, experiments revealed two significant moments: linkage of crayfish-emitted sounds with their behavioural manifestations and evaluation of crayfish acoustic circadian activity under natural conditions. Both of these are of great importance for water quality biomonitoring, because ethophysiological parameters are known to change with environmental deterioration (Bowen and Depledge, 2006). Multi-month circadian activity, expressed by established heart rate, was shown to be well-pronounced for *Astacus leptodactylus* only if they were healthy and undisturbed (Udalova et al., 2009). Thus, it makes sense to suppose sound emission ability, similar to other physiological parameters, in combination with behavioural endpoints, might serve as useful indicators of water status that can be evaluated through crayfish vital factors.

3.1.3. Optical gate

The background locomotor activity or detection of motion events can be implemented by an easily-constructed optical technique (Bolt and Naylor, 1985). An infrared light emitter and photodiode were positioned on opposite walls of the experimental tanks, forming an optical gate (Shuranova and Burmistrov, 2010). These elements (sensors) are located so that movements of as little as a few centimetres by the crayfish would be detected, and when the beam emanating from the emitter is interrupted, it reflects infrared light to the detector. This provides a semiquantitative measure of crayfish locomotor activity (Bojsen et al., 1998).

3.1.4. Biotelemetry

In past and current research, there is separate branch devoted to biotelemetric investigations. We will briefly describe the main telemetry opportunities that, in our opinion, can be beneficial in biomonitoring.

Generally, telemetry is taking measurements remotely (Wolcott, 1995), but use of the prefix "bio" implies measures of biological parameters, both physiological and behavioural (Cooke et al., 2004). The major advantages of all telemetric methods involve not just the remoteness, but the continuity of data collection. Ideally, in biomonitoring, data would be gathered continually with no human manipulation (nontethered animals) after basic preparations. Certain kinds of sensors are used in both biomonitoring and biotelemetry, but descriptions of them, their attachments and uses are best described elsewhere. However, the limits of distance and the assortment of measureable parameters are worthy of discussion.

Radio telemetry

Radio tags emit electromagnetic energy in the radio frequency range, 30 to 300 MHz (Cooke et al., 2013), which carries signals from study objects and are believed to be best used for terrestrial observations because they function better in air, and the electromagnetic waves decay as they travel through water and conductive environments, particularly marine environments. Luckily, for astacologists, who work only with freshwater species, radio telemetry is being successfully applied in both nature and laboratory environments (Bubb et al., 2006; Lowe et al., 2010).

Using radio tags, crayfish movement patterns have been investigated, focused on distribution activities. Studies of note examined (a) crayfish invaders (Bubb et al., 2006), where invasive species were found to overtake indigenous ones under adverse conditions, and a large potential threat for non-native crayfish was found in small reservoirs and tributaries that were inhabited by indigenous species (Buřič et al., 2009); (b) native crayfish (Robinson et al., 2000), where behaviours and habitat selection of disturbed populations was in response to changing environmental conditions; and (c) very unique and rare crayfish (Webb and Richardson, 2004), where home range and movement patterns were investigated.

Acoustic telemetry

Acoustic techniques use transducers to convert electrical energy to acoustic energy that is detected by an underwater hydrophone (Cooke et al., 2013). Up to now, crayfish have not been used as study objects in acoustic telemetry, but Clark et al. (1999) was able to determine agonistic behaviour telemetrically in free-ranging marine crabs, gaining useful insight into the benthic community structure and the relationships between animal density distribution and preying behaviours. Nevertheless, Cooke et al. (2004) emphasised the real benefits of testing acoustic tags on large aquatic invertebrates: obtaining the heart beat, ventilatory rate and the effects of anthropogenic noise on behaviour, even though acoustic techniques are rather uncommon for our subjects, i.e. crayfish (Wolcott, 1995).

Passive integrated transponder (PIT) telemetry

This type of telemetry is based on an integrated circuit chip and a coil antenna that transmits a unique identity code when energised by a low-frequency (125 to 400 kHz) radio signal, enabling use of enough tags to accommodate relevant sample sizes, up to the community

level (Cooke et al., 2013). Using PIT telemetry, Bubb et al. (2006) investigated the extent of space use by invasive crayfish, which is of importance when controlling and managing species movement.

The key application of telemetry in water quality biomonitoring is finding crayfish movement patterns that are directly and tightly related to their physiological status, as well as their feeding and reproductive activities (manifested in heartbeat, ventilatory rate, etc), which are dependent on environmental quality. Thus, it appears that by solving the issues of crayfish ethology, we can address the ecological aspects, which concern not only crayfish, but the condition of the entire water body.

Cooke et al. (2004) remarked that when biotelemetry does not present the appropriate physiological and behavioural information about free-living animals, it often does not fail because of technical mistakes, but because the researcher lacks understanding of how to adjust the technology to the problem, the animal and the environment.

3.1.5. Chelae force measures

This unusual approach was demonstrated by Seebacher and Wilson (2006) and Lowe et al. (2010), whose techniques were to measure the crayfish chelae forces as responses to environmental changes. Chelae-produced forces were measured using a special sensor that consisted of two thin (25 mm x 5 mm x 1 mm) metal plates separated by a thicker (4 mm) pivotal plate, all of which were mounted in a block of wood. Chelae were equipped with strain meters attached to each block using epoxy resin. Each strain meter output was connected to a force-difference measuring circuit (Wheatstone bridge), amplified and monitored using a computerised recording system. Each was calibrated so that strain outputs, in volts, were directly converted into force, in newtons. Equipped with such devices, crayfish readily closed their chelae so that the total force produced by them could be measured.

Initially, such a design was used to assess crayfish vitality reserves during thermal adaptations, but the approach was applied to evaluate crayfish responses to other parameters. Temperature is not related to water contamination. However, it has been reported that the behavioural challenge – fitness – of shore crabs was used as an indicator of poor water quality (Bamber and Depledge, 1997). In practice, after exposure to physical stress, animals collected from environments with greater contamination levels had significantly higher heart rates than those from better environmental conditions. Measuring the chelae forces might be used with a similar intent, because when impaired by negative environmental impacts, crayfish would theoretically demonstrate weaker chelae forces.

3.2. Biomonitoring

Biomonitoring is a quantification of the relationships between anthropogenic disturbances and biological indicators, detected through their bio-ecological responses. Bioindicators of some species readily reflect the abiotic or biotic state of an environment and represent the impact of environmental change in the habitat, community and entire ecosystem (Hodkinson and Jackson, 2005).

We believe the reactions of bioindicators depend on anthropogenic activities, and nowadays, these activities are reactions to those preceding human activities. And, incidentally, these reactions are far from positive in many cases. However, biomonitoring is carried out for exactly the purpose of helping each other: humans examine the environment, its inhabitants respond according their feelings of well-being, and humans suggest how to help them, if necessary.

Monitoring and tracking behaviour is fine, but it does not reveal the entire status of the crayfish. For example, movement, as an expected reaction to some stimuli, might not be demonstrated at all. However, heart and ventilatory rates can vary greatly, even when the crayfish is stationary. When heartbeat and respiration are within normal ranges, an undisturbed state is assumed (Depledge and Galloway, 2005). Of course, "normal" here refers to an explicit range in accordance with the physiological rhythm appropriate for the day time and relative conditions, such as light vs. dark or warm vs. cold (Chabot and Webb, 2008; Styrishave and Depledge, 1996).

Paaragraphs 3.2.1. and 3.2.2. will consider measures of crayfish heart and ventilatory rates. These basic physiological parameters can be measured using two types of approaches: invasive or noninvasive biomonitoring.

3.2.1. Measuring heart rate

Invasive measurement

Invasive biomonitoring implicates direct insertion or implantation of measuring wires, which end with sensors or electrodes, to "connect" an animal to a gauge. In this case, the crayfish plays the role of some kind of gauge as well, but its physiological data are first passed through auxiliary devices to convert crayfish signals into a "readable" value for the researcher.

Impedance pneumography and electrocardiography

Impedance pneumography (IPG) was the first technique applied to measure the heart rates of large crustaceans, and it allowed observations to be made with very little disturbance to the normal (post-surgery) activities of the animals. It was initially applied to a bivalve mollusc *(Mya arenaria)* as described by Hoggarth and Trueman (1967) and modified when used on *Crangon crangon* (Dyer and Uglow, 1977), *Carcinus maenas* (Cumberlidge and Uglow, 1977), *Palaemon elegans* (Morris and Taylor, 1984) and bivalve molluscs of genus *Mytilus* (Braby and Somero, 2006). With the aid of an impedance pneumograph, a low oscillatory current (2 μA) could be transmitted between two tiny wires, serving as electrodes when:
1. Each wire is attached to one valve of a clam to register a voltage (the so-called impedance between electrodes) caused by any valve movement.
2. The heart electrode is hooked over the dorsal posterior margin of a shrimp or crab cephalothorax, and the scaphognathite electrode is hooked over the anterior margin of the prebranchial chamber.
3. A silver electrode is implanted in the pericardium of *Palaemon elegans* by drilling a small hole in the carapace immediately dorsal to the heart, and fixed in place using quick-setting cyanoacrylate adhesive, after which, a second external electrode is wound around the first, immediately above the point of entry into the carapace, thus maintaining electrode geometry and making it insensitive to the prawn's movements, a significant advantage in biomonitoring necessary in each method.
4. After drilling a small hole in each mussel valve immediately over the pericardial space, they are individually inserted and held in place by quick-drying surgical glue, thus producing an analogue impedance signal that could be converted to a voltage signal and digitally recorded for further analysis.

For some reason, this method has never been tested on crayfish species, but despite it being quite primitive, we believe it could be applied for basic heart rate measurements in crayfish as well.

Another way to invasively measure cardiac activity is by using *electrocardiography (ECG)*. This technique was primarily tested on crabs *Cancer magister* and *Cancer productus* (Florey and Kriebel, 1974) and much later tried on crayfish *Cherax tenuimanus* (Villarreal, 1990), lobster *Homarus americanus* (Chabot and Webb, 2008), shrimp *Macrobrachium rosenbergii* (Chung et al., 2012), but since 2000 is actively in use with crayfish: *Orconectes australis packardi* (Li et al., 2000), *Procambarus clarkii* (Listerman et al., 2000), *Cherax destructor* (Goudkamp et al., 2004), *Procambarus clarkii* and *Orconectes australis packardi* (Bierbower et al., 2013).

Recording ECG has changed little over the years and species, and is described in the above sources. To obtain an electrocardiogram, a crayfish is equipped with two insulated stainless steel wires placed under the dorsal carapace directly over the heart a few days (3 days is optimal) prior to experimentation. They are inserted through holes drilled in the carapace, and are then cemented in place using instant cyanoacrylate adhesive with an additional accelerator application. All cardiac signals are determined using an impedance detector, which measures the dynamic resistance between the stainless steel wires (i.e., instantaneous voltage emergent between applied electrodes), which is then recorded using a computer. The heart rate is determined using direct measurement with a window discriminator, which measures a running average of instantaneous events. The values are converted to beats per minute and stored for later analysis (Bierbower et al., 2013; Chung et al., 2012).

Electromyogram

Electromyograms (EMGs) from unrestrained crayfish were made by Tsuchida et al. (2004) and Chikamoto et al. (2008). As described by Tsuchida et al., a special transmitter is mounted on the ventral side of the carapace and affixed to the outer surface by an adhesive. Transmitter size varies with the number of channels. Either dual- or quad-channel transmitters can be used, depending on the size of the crayfish, and one photodiode is placed at each corner of the experimental aquarium (0.3 m wide, 0.4 m deep, 0.15 m high). This experimental area is not limited by optical signal extinction, and it is possible to record the signal wherever the crayfish is in the experimental arena. The signal can be received up to 0.5 m from the transmitter; however for transmission over longer distances, some modification of this system would be needed, such as using more photodiodes inside the same transmitter to expand the experimental area. This principle can be defined as a separate approach, termed underwater *optical telemetry*.

Chikamoto et al. (2008) obtained EMGs of the four muscles of the thoracic legs of crayfish and simultaneously video-recorded leg movements to quantitatively characterise walking behaviour, either chemically initiated or spontaneous. Extracellular recording from one of the walking leg muscles was accomplished using a pair of silver wires inserted into the relatively immobile region of the muscle through fine holes drilled in the cuticle. The holes were sealed and the wires glued to the cuticle. Thus, electrical signals from activated and non-activated crayfish muscles were obtained.

Though a better signal might now be obtained through direct crayfish wiring, it is nevertheless important to have crayfish nontethered, as with the underwater telemetry system, and the benefit of this requirement is evident: no movement restrictions, no waiting for recovery of animals post-handling, fewer effects on the crayfish by technical loading and more reliable responses to tested stimuli.

Noninvasive measurement

Currently, the major body of scientific publications is devoted to noninvasive approaches aimed at measuring physiological parameters in living organisms, and the crayfish is no exception. Noninvasive biomonitoring enables minimisation of disturbances to the animals and manipulation of time, theoretically providing researchers with the most relevant physiological data.

Photoplethysmography (PPG)

Despite having a modern title and being widely used, PPG has its roots in the 1980s, when it was introduced by Depledge (1984). The beauty of this approach is that it can easily be adapted to a variety of crustaceans, bivalves, gastropods and even such terrestrial arthropods as spiders. However, the illumination source and transmission cable can vary, influencing the price of the sensor and its functional capabilities.

When initially presented, the pulse PPG sensor consisted of two small, low-intensity bulbs and a phototransistor. To measure the cardiac activity of decapod crustaceans, the sensor had to be attached to the outer carapace over the heart and affixed by a quick-drying, water resistant and environmentally friendly adhesive. The low-intensity light emitted by the bulbs passed through the carapace into the animal's pericardium. When cardiac muscle contracted, the pericardium reflected a certain part of the light, which fell onto a sensitive phototransistor, which in turn transformed the light into a proportional voltage change (light intensity difference), sent to a storage device, initially an oscillograph (Depledge, 1984).

The "trick" in using such easily-obtained differences between sent and reflected light intensities is easily explainable: a widely used method for the detection of changes in blood volume or any other fluid, such as hemolymph, as in the case of crayfish, caused changes in microvascular tissues (Allen, 2007), and the PPG technique did not use the volume itself, but recorded the fluctuation associated with blood (or hemolymph) filling vessels, and this fluctuation was counted as one pulse or beat (Lu et al., 2009).

The next development in PPG, which was a great event in the research arena, was the public introduction of a *computer-aided physiological monitoring system (CAPMON)* for continuous, long-term recording of cardiac activity in select invertebrates by Depledge and Andersen (1990). The same principle as in Depledge (1984) was used as the basis, but with technical modifications of the transducer, which consisted of a near-infrared light-emitting diode and a phototransistor detector. The elements were mounted parallel, facing in the same direction. The phototransistor detected variations in infrared light intensity and generated a current, which was dependent on the reflected light, and then was filtered, amplified and screened using a computer interface. The transducer was connected to the computer interface by fine, flexible wire so that the animal was virtually unrestrained within its aquarium. Additionally, direct observation of heart beats via ECG confirmed that beating concurred with the peaks obtained by PPG, so that heart activity could be monitored in several individuals simultaneously, 24 hr per day, over prolonged periods. The maximum duration of an observation period was most likely limited by the animal's moulting cycle (in the case of crayfish, crab, shrimp and lobster), where the transducer remained with the old carapace left by the moulted animal.

Because perfection does not have limits, in the following years, this transducer was modified for heart rate counting. Thus, Depledge et al. (1996) reported the development of an automated interpulse-duration assessment (AIDA) technique, which enabled a more detailed analysis of measured heart rate variability and disturbances. Detailed analysis revealed periods of regularity and irregularity in the cardiac activities of crayfish, however without

details, the mean heart rate in the two periods appeared to be equal. The AIDA technique took this peculiarity into account and made possible continuous interpulse intervals monitoring, beat regularity assessment and identification factors caused by beating irregularities.

In contrast to this modification, Burnett et al. (2013) suggested simplifying the CAPMON technique, arguing that heart rate irregularities had to be identified. Instead of automatically calculating the average heart rates, their updated method enabled signal amplification and direct saving of the "raw" data. Maintaining the true shape of the cardiac wave was beneficial because each organism was likely to exhibit a unique heartbeat pattern that might otherwise be misinterpreted by an automatic counting circuit, like that seen on the CAPMON system. Additionally, keeping heartbeat signals raw might allow identification of bradycardia (McMahon, 1999), which was potentially caused by a worsened ambient state, including hypoxicity, which occurred because of oxygen depletion followed by strong environment deterioration.

In spite of the initial testing and development of PPG and related optic techniques on crab models, it is hardly possible to imagine fresh water monitoring with no consideration of these pivotal species. With the aid of the CAPMON system, it was possible to begin using crayfish for their direct vocation: indicators of water quality and its impact on living organisms (Styrishave et al., 1995). With minimal harm but maximal efficiency, up to eight crayfish were observed simultaneously, combining a few factors, and recording all the behaviours and physiological states for *Astacus astacus* (Bojsen et al., 1998) and *Pacifastacus leniusculus* (Bloxham et al., 1999).

Starting in 2000, a new biomonitoring technique, expanded by Fedotov et al. (2000), appeared. Having in its basis the ideas of the CAPMON system, developments in heart rate measurement and assessment were introduced so that the system finally acquired the name of the *fibre-optic method for registration and analysis of cardiac activity of benthic invertebrates*, the System for Industrial Biological Water Quality Monitoring (SIBWQM; Kholodkevich et al., 2008). The principle is the same (measure fluctuations of the scattered and reflected infrared light), but sources of light were replaced by low-power semiconductor lasers, and transmitting this light was accomplished using fibre-optic cables. To avoid repetition, but not skip important details, the operation steps are delineated:

1. An infrared light beam, formed in the laser fibre-optic photoplethysmograph is transmitted to the animal through a thin optical fibre.
2. A small sensor, connected to additional optical fibre, is attached to the carapace and detects scattered light in the heart area.
3. The optical signal is modulated by heart muscle contractions.
4. After appropriate amplification and filtration, the analogue signal is converted to a digital form and transmitted to a computer (Fedotov et al., 2000, 2006).
5. The resulting photoplethysmogram can be further analysed by mathematical and statistical methods, including profitably applied variation pulsometry (Kholodkevich et al., 2008).

This approach deserves a special honour because its application was, and still is, predominantly focused on the various aspects of astacological research, from the peculiarities of crayfish cardiac activity in various functional states (Fedotov et al., 2002) and in varying natural conditions (Sladkova et al., 2006; Udalova et al., 2009, 2012) to chemical stress assessments (Kozák et al., 2009, 2011; Kuznetsova et al., 2010) as well as insight into cardio-biochemical parameters (Sladkova and Kholodkevich, 2011). The only point missing when mentioning this approach is the lack of applications involving ventilatory activity monitoring. This was accomplished using other techniques.

3.2.2. Measuring ventilatory rate

Respiratory activity is as vital as that of the cardiovascular system in crayfish. Although it is meaningless to separate these functions, it is quite obvious that when in water, crayfish breathe in a way that exploits contact with substances, and if they are chemical, not visual, disturbances, the ambient status the cardiovascular function is adapted. This does not mean that an increase in the ventilatory rate precedes one in the heart rate. It is more likely that if a crayfish is disturbed, the cardiac system becomes more active, demanding a better oxygen supply, and then the ventilatory rate would rise. Vogt (2002) highlighted crayfish ventilation frequency and volume as very much dependent on the status of activity, water temperature and oxygen content of the water. But these conditions can evidently be extended to additional factors, predominantly, but not limited to, human causes (i.e., release of chemicals, etc.) that affect water quality. Burmistrov and Shuranova (1996) demonstrated that ventilatory rate reflects the crayfish functional state, similar to the heart rate, correlating with, and indicating, environmental changes. Thus, the ventilatory rate allows indexing the characteristic state of the animals (which implies the consequent reflection of the environmental ambient state).

Invasive measurements

When crayfish breathe, water and dissolved gases are drawn through the gills with rhythmic scaphognathite (SG) beating. Actually, this beating reflects the crayfish ventilatory rate, and the same procedure used for ECG can be used to obtain an electroscaphognathitegram (ESG). To record ventilatory activity, the cleaned ends of two enamelled copper wires are placed under the carapace, near the anterior end of the scaphognathite chamber and near the carapace The wires are cemented in place using cyanoacrylate ester and accelerator and are additionally affixed to the dorsal carapace surface (Schapker et al., 2002; Shuranova and Burmistrov, 2002). To record the ESG, the wires from the crayfish are connected to an impedance detector. The ventilatory rate is seen in the dynamic resistance between the two stainless steel wires, and these signals are recorded and screened on-line (Bierbower and Cooper, 2009).

Noninvasive measurements

The principle applied in PPG for monitoring cardiac activity can also be applied to measure ventilatory rate. By attaching the photodetector on the prebranchial surface of the carapace under the SG periodic ventilatory movements can be monitored (Depledge, 1984).

Bini and Chelazzi (2006) showed an example application of this technique for SG PPG monitoring in crayfish, using a flat sensor placed latero-ventrally on the gill chamber. This was the only noninvasive method mentioned for crayfish ventilatory rate monitoring in the literature. However, new studies can appear at any time, bringing new contributions into the biomonitoring bank.

3.3. Monitoring combined parameters (physiologically-ethological)

In spite of the simpler organisation compared to vertebrate organisms, the crayfish nervous system is responsible for the same vital processes: heartbeat and vascular pressure, respiratory and energy exchange, digestion and metabolism (Vogt, 2002). So there is no surprise that introducing stress stimuli can trigger an entire chain of etho-physiological reactions: stress-stimulus is introduced → fan organs create water flow → chemical signal is obtained → matter is breathed in → heart rate is accelerated → oxygen consumption is increased → ventilatory rate

rises → locomotory activity is manifested → total stress state is established and pronounced. The relative simplicity of crayfish makes the responses easier to manifest. At the same time, they possess enough complexity to express complex reactions well. If the crayfish is disturbed (by water deterioration, for example), numerous physiological and behavioural changes would occur, and it is good, from a biomonitoring standpoint, if we can observe at least two of them.

The most rational combination would be a vital physiological characteristic (intrinsic for crayfish but needed to measure and screen) paired with an ethological trait (manifested by crayfish and possible to observe visually). Quite a large body of publications has been devoted to this issue. Bojsen et al. (1998) successfully combined cardiac and locomotor activity monitoring and found them to be tightly related to one another, as well as to the ambient temperature and light intensity. Bini and Chelazzi (2006) carried out a double physiological study, where it was shown that heart and ventilatory rates were correlated and decelerated under unfavourable conditions (presence of copper). Crayfish heart rate, locomotor activity and oxygen consumption were shown to be circadian, with significant increases in all parameters during the night (Styrishave et al., 2007). Underwater sound emissions by crayfish were studied along with behavioural observations, and it was shown that crayfish produce more sounds at night or when contacting other crayfish (Buscaino et al., 2012). In addition, the background locomotor activity was studied in relation to SG activity, and it was revealed that even physically stationary crayfish are wakeful and continuously monitoring their environments (Shuranova and Burmistrov, 2010). Finally, behavioural manifestations were examined in relation to environmental changes and were combined with cardiac activity monitoring by Bierbower et al. (2013).

4. BAD MATTERS IN PLAIN WATER

Pollutants in water, even when in low doses, can prevent normal crayfish senses, which play huge roles in the animals' lives (Bergman and Moore, 2005). As mentioned, crayfish reactions are quite complex chains of sub-responses, thus an imbalance in one unit can cause dysfunction of the whole organism. Moreover, numerous pollutants can affect other vital functions of crayfish and any other aquatic organisms. Blaxter and Hallers-Tjabbes (1992) gave a detailed list of aquatic pollutants and the harms they cause for aquatic organisms. Among them are heavy metals, organic industrial pollutants, acidifiers, heat, excesses of nitrogen- and phosphorus-based nutrients, mechanical disturbances and even radioactive pollution. From the examples given and the described techniques, it is seen that the effects on crayfish of many of these pollutants have been examined. Also, many studies were conducted using biomonitoring approaches, thus allowing continuous exploring and monitoring of pre-treatment and post-treatment crayfish states. Moreover, studies of bioaccumulation rates are also sometimes necessary (Table 1).

5. EXPERIENCE, APPLICATION, CONCLUSIONS

It is valuable to study the heart rate, not just in real time, to be able to assess an immediate crayfish response to environmental changes. Additionally, heart rate monitoring should be continuous, and conducted over long periods. The reasons, though quite obvious, must be kept in mind:
1. To make sure there are healthy animals available for experiments.
2. To determine the impacts of numerous environmental conditions besides the chemical and negative ones, such as physicochemical parameters (temperature, pH, dissolved oxygen).

3. To predict the overall environmental stress when it is accumulated and summed.

We might be able to prevent these unwelcome stress excesses by continuously monitoring the environment. Otherwise, if the stress reaction was an experimental aim, we might intensify the crayfish reaction from one condition to another, instead of evaluationing some overall, and uncertain, attained heart rate (or another characteristic). Using continuous monitoring, it is possible to draw crayfish reactions and to distinguish chemical and natural stress sources.

We saw that a wide range of crayfish species were tested worldwide in variable biomonitoring aspects (Table 2), but this does not mean that each of them would be suitable for any study that we might try to establish. Regardless of whether we want to or not, each approach has to be newly reset, or "over-calibrated," for each individual purpose. For example, the methods verified on red-swamp crayfish may not give good results if tested on signal crayfish, but could bring relatively satisfactory outcomes on the red-claws or other warm-water species. Though many difficulties are faced up front, some turn into advantages, and each approach can be fitted according to the available tools if thought out in advance. For example, if water quality monitoring was initiated in a country with varying seasonal temperatures over the year (likely in a majority of cases), at least two crayfish species that would give adequate reactions under similar conditions should be kept ready. Of course, the "hot" question of manipulation with invasive species will arise, but rationally, the problem of non-native species interacting with native ones can be carefully avoided.

Generally, this is a great opportunity to monitor such vital functions as heartbeat and respiration, and to do so while avoiding traumatising the animals and also involving them in such critical process as environmental quality control.

Despite quite many promising techniques discussed in this chapter, only a handful were shown to be useful for biomonitoring in its true sense, and the remainder have not been tried in wide arenas as yet. Forever highly optimistic, we suggest that this is quite positive as well, because there is big space for many interesting investigations as yet, and applications of existing knowledge could find many beneficial implications in the near future.

Acknowledgements

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Table 1. *Physicochemical stressors and related crayfish parameters which these stressors impact.*

Stimulus	Study subject and main outcomes	Reference
Chemical		
Ammonia	**Heart rate changes.** Concentrations above 5 mg l^{-1} have significant stimulatory effect on crayfish heart rate.	Bloxham et al., 1999
Carbon dioxide	**Heart and ventilatory rates.** There is a CO^2 mediated effect on both the cardiac and ventilatory systems.	Bierbower and Cooper, 2010
Chlorides	**Cardiac activity.** Concentrations of NaCl higher 3200 mg l^{-1} cause a clear increase of crayfish heart rate, but chlorides have positive effect on cardiac activity if applied prior to nitrite exposure.	Kozák et al., 2009, 2011
Hydroquinone	**Cardiac activity and hemolymph biochemical parameters.** Hydroquinone (1000 mg l^{-1}) has a temporary increase of the heart rate and no marked effect on hemolymph total protein when applied for short-term, while at long action, hydroquinone causes tachycardia and significant protein decrease.	Kuznetsova et. al., 2010; Sladkova and Kholodkevich, 2011
Metals	**Survival, heart and ventilatory rates.** Depending on progressive concentration (50 to 200 mg l^{-1}) and longer exposure, heart and ventilatory rates tend to slow down by waterborne copper, while stress effect is reduced if crayfish were pre-exposed to lower copper concentration; increasing copper leads to crayfish higher mortality.	Bini and Chelazzi, 2006
	Circadian rhythm of the heart rate. Mercury (0.1 mg l^{-1}), copper (8 mg l^{-1}) and sodium chloride (1400 mg l^{-1}) cause disruption in crayfish circadian cardiac activity, that is reflected in depressed heart rate during the night.	Styrishave et al., 1995
Nitrite	**Hemolymph nitrite and glucose, total hemocyte counts.** Hemolymph nitrite increased significantly with higher nitrites level (9 to 25 mg l^{-1}) in water, but increased less with addition sodium chloride (1000 mg l^{-1}), while hemolymph glucose elevated regardless of water nitrite concentrations and did not increase if chloride was present; at higher nitrite concentration, hemocyte counts decreased.	Yildiz and Benli, 2004
Own, conspecific odours	**Behavioural and heart rate changes.** When crayfish were exposed to their own and conspecific odours, the behavioural changes measured by bodily movements were not demonstrated, but heart rate could be significantly elevated.	Li et al., 2000
Predatory fish, conspecific odours	**Prey-predator behaviour.** Behavioural response (as indicated by the time spent feeding, in locomotion and in the lowered posture) of crayfish is better pronounced when they are exposed to conspecific odour rather than to predatory fish odours.	Gherardi et al., 2011
Serotonin	**Social interactions and heart rate.** Crayfish increase the heart rate during an interaction to establish social status, while progressive level of serotonin (100 nM to 10 μM) increase crayfish heart rate for hours.	Listerman et al., 2000

Physical

Acoustic stimulus	**Immune mechanism and behaviour.** Exposure to the noise produced significant variations in haemato-immunological parameters as well as a reduction in agonistic behaviour.	Celi et al., 2013
Illumination	**Cardiac and motor activities.** Infrared or dim red lighting did not result in an elevated heart rate but exposure to white light resulted in an increase in heart rate not only during the walking phases but between moving as well.	Li et al., 2000
Physical load	**Cardiac activity.** Ability to maintain the heart rate high during physical load (1 h suspension) indicates crayfish tolerance to short term chemical exposure.	Kozák et al., 2011
	Circadian cardiac rhythmicity. Weak reactions to physical stress (inability to maintain increased levels of heart rate for 1 h of exposure) reflects instability of excitation and inhibition processes in the nervous regulation of cardiac activity, a demonstrative indicator of crayfish stress condition.	Udalova et al., 2012
Temperature	**Cardiac and locomotor activities.** Heart rate and locomotor activity correlated positively, while both parameters decreased at lower temperatures.	Bojsen et al., 1998
	Survival and heart rates. Crayfish remained responsive to sensory stimuli and survived with either rapid or slow changes in temperature (21 to 5 °C); the acute rapid drop in temperature resulted in a substantial reduction in heart rate, while it decreased gradually at chronic changes of water temperature.	Chung et al., 2012
	Activity and chelae force. Examined crayfish species (*Euastacus sulcatus*) used neither thermoregulatory behaviours (changes of locomotion activity) nor physiological strategies to deal with changes in environmental temperature (20 to 10 °C).	Lowe et al., 2010
	Oxygen consumption, activity and heart rate. Temperature is the principal factor influencing heart rate, which was significantly lower at lower temperatures (18 °C). Higher temperatures caused more intensive oxygen consumption and locomotion, while at 26 °C the stress effect was evident.	Villarreal, 1900
Waterborne vibrations	**Behavioural body movement and heart rate.** A drop of water falling in front of the animal caused a brief increase in heart rate which lasts a few minutes, while crayfish were not responsive behaviourally; a more dramatic duration in an elevated heart rate was induced after a dropped pebble, and alterations in the water level also resulted in brief increase of heart rate, but behavioural response was not demonstrated in all cases.	Li et al., 2000

Table 2. *Geographically-specific crayfish diversity and techniques they were tested with.*

Species	Country	Technique	Reference
Astacopsis gouldi	Australia	Radio telemetry	Webb and Richardson, 2004
Astacus astacus	Denmark	Optical gate, PPG	Bojsen et al., 1998
			Styrishave et al., 2007
		PPG	Styrishave et al., 2005
			Styrishave and Depledge, 1996
	Russian Federation	PPG	Fedotov et al., 2000, 2002, 2006
			Sladkova et al., 2006
Astacus leptodactylus	Russian Federation	PPG	Fedotov et al., 2000
			Kozák et al., 2009, 2011
			Kuznetsova et al., 2010
			Sladkova et al., 2006
			Sladkova and Kholodkevich, 2011
			Udalova et al., 2009, 2012
		ESG	Shuranova et al., 2003
	France	ECG	Dejours and Beekenkamp, 1977
Austropotamobius pallipes	England	Radio telemetry	Robinson et al., 2000
Cherax destructor	USA	Camera-based tracking	Basil and Sandeman, 2000
	Australia	ECG	Goudkamp et al., 2004
		Camera-based tracking	Patullo et al., 2007
		Chelae force measurement	Seebacher and Wilson, 2006
Cherax tenuimanus	Australia	ECG	Villarreal, 1990
Eustacus sulcatus	Australia	Chelae force measurement, Radio telemetry	Lowe et al., 2010
Orconectes australis packardi	USA	ECG, Video recording	Bierbower et al., 2013
			Li et al., 2000

Species	Country	Method	Reference
Orconectes limosus	Czech Republic	Radio telemetry	Buřič et al., 2009
Orconectes rusticus	USA	Camera-based tracking	Bergman and Moore, 2003
			Panksepp and Huber, 2004
Orconectes virilis	USA	Camera-based tracking	Bergman and Moore, 2003
Pacifastacus leniusculus	UK	PPG	Bloxham et al., 1999
	Denmark	Optical gate, PPG	Styrishave et al., 2007
Procambarus clarkii	USA	ECG	Chung et al., 2012
			Listerman et al., 2000
		ECG, ESG	Bierbower and Cooper, 2009
			Cooper et al., 2011
			Schapker et al., 2002
	Italy	PPG, SG PPG; Video tracking, Passive acoustic monitoring	Bini and Chelazzi, 2006; Buscaino et al., 2012
	Russian Federation	PPG	Fedotov et al., 2006
			Sladkova et al., 2006
	France	Camera-based-tracking	Jamon and Clarac, 1995
Procambarus cubensis	Russian Federation	Camera-based tracking	Burmistrov and Shuranova, 2010
			Shuranova and Burmistrov, 2009
		ESG, Optical gate	Shuranova and Burmistrov, 2002, 2010

Research Article

Accumulation of Heavy Metals in Crayfish and Fish from Selected Czech Reservoirs

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To evaluate the accumulation of aluminium, cadmium, chromium, copper, lead, mercury, nickel, and zinc in crayfish and fish organ tissues, specimens from three drinking water reservoirs (Boskovice, Landštejn, and Nová Říše) and one contaminated site (Darkovské moře) in the Czech Republic were examined. Crayfish hepatopancreas was confirmed to be the primary accumulating site for the majority of metals (Cu > Zn > Ni > Cd > Cr), while Hg and Cr were concentrated in abdominal muscle, and Al and Pb were concentrated in gill. Metals found in Nová Říše specimens included Cu > Zn > Ni and those found in Boskovice included Zn > Hg > Cr. Cd concentrations were observed only in Landštejn specimens, while contaminated Darkovské moře specimens showed the highest levels of accumulation (Cu > Al > Zn > Pb). The majority of evaluated metals were found in higher concentrations in crayfish: Cu > Al > Zn > Ni > Cr > Cd > Pb, with Hg being the only metal accumulating higher in fish. Due to accumulation similarities of Al in crayfish and fish gill, differences of Hg in muscle, and features noted for the remaining metals in examined tissues, biomonitoring should incorporate both crayfish and fish to produce more relevant water quality surveys.

1. Introduction

Maintaining suitable freshwater quality is essential for both aquatic and terrestrial life. Monitoring based on relevant bioindicators provides useful data for evaluation of environmental status [1]. Although the hazards of water contamination by heavy metals are well known, it remains an issue due to expanding industrial development, including mining activities [2]. Macroinvertebrates are frequently suggested as bioindicators for monitoring changing water conditions in areas of potential contamination [3]. In practice, crayfish are of particular importance for biomonitoring studies [4], being the keystone species in most ecosystems in which they occur [5], and, most importantly, can tolerate polluted environments and reflect pollution levels due to accumulation of respective elements in their tissues [6]. Algae and fish are also successfully employed in biomonitoring programmes, although algae can sometimes be difficult to identify, while fish are mobile and can potentially avoid contaminated areas

[3]. Water body contamination can be assessed by the quantity of selected elements accumulated in target organisms and their tissues. Much study had been devoted to the assessment of heavy metal bioaccumulation in aquatic biota [2], including crayfish [7] and fish [8], with each bioindicator having its merits [9]. Quantification of bioaccumulation of hazardous chemicals as indicated by their concentrations in organ tissue is the basis of biomonitoring [10]. While crayfish are useful as bioindicators of contamination, they are also a valuable food source [11], making monitoring of organ tissue metal concentrations relevant to both animal and human health.

This primary objective of this study was to survey metal concentrations in crayfish as representative biota of drinking water reservoirs and to relate these results to data on metal accumulation in fish from the same areas. The establishment of such relation was important for underlining specific attributes of selected elements accumulation for examined reservoirs and resident species.

2. Materials and Methods

2.1. Studied Localities. Heavy metal content in organ tissue was assessed in crayfish from three Czech water supply reservoirs: Boskovice (South Moravian Region; $49°29'50''$N, $16°41'59''$E), Landštejn (South Bohemian region; $49°1'21''$N, $15°14'30''$E), and Nová Říše (Vysočina region; $49°29'50''$N, $16°41'59''$E). A fourth reservoir known to be contaminated with heavy metals, Darkovské moře (Moravian-Silesian Region; $49°49'56.935''$N, $18°33'10.230''$E), was used as contaminated. The contaminated reservoir is a lowland (maximum surface area 32 ha, depth 28 m) flooded by ground waters in the 1990s, located in a region highly affected by coal mining. The shoreline and vicinity are formed by gangue deposits. The reservoir is currently used for recreation.

2.2. Crayfish Sampling. Crayfish were caught in baited traps from June to November 2008: Boskovice on 10 June, Nová Říše on 19 June, Landštejn on 19 June and 9 July, and Darkovské moře on 7 and 12 November. For each site, 10 intermolt males were selected from trapped noble crayfish, *Astacus astacus* (L. 1758). Crayfish were grouped based on carapace length (CL) and the postorbital carapace length (POCL) to the nearest 0.1 mm and total weight (TW) to 0.1 g. Crayfish measurements from test sites were CL = 48.4 ± 1.9 mm, POCL = 36.5 ± 1.6 mm, and TW = 28.7 ± 3.3 g, and for contaminated locality: CL = 59.9 ± 2.2 mm, POCL = 41.9 ± 1.7 mm, and TW = 39.5 ± 6.7 g. The crayfish from the Darkovské moře were significantly larger than those from drinking water reservoirs, where crayfish size did not significantly differ.

2.3. Metals Analysis. Prior to dissection, the selected crayfish were immediately immersed in liquid nitrogen or, for specimens from the control site, subjected to short-term freezing, and samples of abdominal muscle, hepatopancreas, and gill were obtained. Abdominal muscle and hepatopancreas were analysed for zinc, cadmium, lead, copper, nickel, chromium, and mercury content. In the crayfish specimens from Darkovské moře, nickel was not measured, and hepatopancreas, abdominal muscle, and gill were analysed for zinc, cadmium, lead, copper, chromium, and mercury. Gills of crayfish from all sites were also analysed for the presence of aluminium. Because of the small amount of gill tissue available for aluminium analyses, tissue samples from two randomly selected individuals were paired into five samples for each test locality, while gill samples of crayfish from Darkovské moře were analysed individually.

Metal analyses were carried out in accredited laboratories of State Enterprise Povodí Moravy (Brno, Czech Republic) using operating procedures according to EN International Organization for Standardization (ISO) standards.

Al, Cd, Cr, Cu, Pb, Ni, and Zn determination was performed according to the method DIN EN ISO 17294-2:2005 (water quality, application of inductively coupled plasma mass spectrometry, ICP-MS) using the inductively coupled plasma mass spectrometer Perkin Elmer Elan DRC-e (Perkin Elmer, Waltham, MA, USA). Detection limits were as

Table 1: Biometric parameters (TL: total length, W: weight) of fish ($n = 16$) from drinking water reservoirs.

Reservoir/Species	TL, (mm)	W, (g)	Age, (yr)
Boskovice			
Abramis brama	350	400	4
Scardinius erythrophthalmus	230	120	6
Scardinius erythrophthalmus	220	100	5
Perca fluviatilis	190	90	4
Rutilus rutilus	270	190	5
Tinca tinca	440	850	5
Landštejn			
Abramis brama	510	1600	8
Perca fluviatilis	280	250	5
Perca fluviatilis	330	400	7
Rutilus rutilus	330	380	5
Tinca tinca	450	1100	11
Nová Říše			
Abramis brama	400	670	5
Perca fluviatilis	330	450	6
Perca fluviatilis	380	980	9
Sander lucioperca	710	3300	7
Tinca tinca	400	850	5

follows: 5 mg kg^{-1} dry weight for Zn and Al, 0.5 mg kg^{-1} for Cr, Cu, Pb, and Ni, and 0.05 mg kg^{-1} for Cd. The samples were freeze-dried using Christ Alpha 1-2 lyophilizer, grinded in Retsch spherical mill, and decomposed with Milestone Ethos-1 microwave decomposition apparatus (Czech technical standards ČSN EN 13657, solid matrices samples preparation, screening, and skeleton determination).

Samples for Hg determination were processed according to the Czech technical norms, ČSN 757440 (determination of total mercury by atomic absorption spectroscopy), on AMA-254 Analyzer (Altec, Prague, Czech Republic) by direct measurement, without microwave decomposition, with detection limit of 0.01 mg kg^{-1}. Metals concentrations are expressed on the dry weight (dw) basis.

2.4. Metal Detection in Fish. State Enterprise Povodí Moravy provided data on Zn, Cd, Pb, Cu, Hg, and Al in muscle of the common bream *Abramis brama* (L., 1758), common rudd *Scardinius erythrophthalmus* (L., 1758), European perch *Perca fluviatilis* (L., 1758), pikeperch *Sander lucioperca* (L., 1758), roach *Rutilus rutilus* (L., 1758), and tench *Tinca tinca* (L., 1758) of different size and age (Table 1). Analytical methods were similar to those used for crayfish. The assessment was conducted in the same sites, with the exception of Darkovské moře, at approximately the same time period in 2008: Nová Říše, 19 June; Boskovice, 9 July; Landštejn, 30 September.

2.5. Statistical Analyses. Crayfish biometric parameters were tested for data normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene test) and compared among sites

Table 2: Statistical evaluation of crayfish biometric parameters.

Test	CL	POCL	TW
$KW - H$ (3, 40)	22.79	22.08	18.02
P	<0.05	<0.05	<0.05

by nonparametric Kruskal-Wallis test (Table 2), because of heterogeneity of variances.

For statistical analysis, metal concentrations below the detection limit were replaced with the mentioned detection limits. Differences in metal content of crayfish tissue among localities were evaluated using the nonparametric Kruskal-Wallis test (Tables 3 and 4), followed by multiple means comparison of all groups as a post hoc test.

To evaluate whether the measured metals showed greater accumulation in hepatopancreas or abdominal muscle of crayfish, the nonparametric Wilcoxon test for matched pairs was conducted (Table 5). To compare accumulation in abdominal muscle, hepatopancreas, and gill of specimens from the contaminated site, the Friedman ANOVA test was applied (Table 6). Aluminium accumulation in crayfish gill from all localities was evaluated using the Kruskal-Wallis test (Table 7) followed by multiple comparisons of mean ranks for all groups.

The significance level for all tests was ≤0.05, while statistical evaluation was conducted using STATISTICA.10 software for Windows (StatSoft, Czech Republic). Data are presented as mean values ± standard deviations.

Biometric parameters of fish and metal concentration in fish muscle were not statistically compared due to variation in age and size of specimens.

3. Results

3.1. Selected Metals Content in Crayfish Hepatopancreas. Zinc content was significantly lower in the specimens from the Landštejn Reservoir, 100.29 ± 34.98 mg kg^{-1}, with concentrations in specimens from other reservoirs nearly double that value (Table 8). Landštejn specimens showed the highest Cd content, 7.31 ± 2.56 mg kg^{-1} (Table 8). Pb content was below the detection limit (<0.50 mg kg^{-1}) for all sites except the Landštejn Reservoir at 0.82 ± 0.35 mg kg^{-1}. The lowest Cu content was detected in samples from Landštejn, 30.41 ± 32.22 mg kg^{-1}, with a higher concentration in the Nová Říše Reservoir (410.10 ± 154.70 mg kg^{-1}) and significantly higher concentration at the contaminated site (794.70 ± 234.74 mg kg^{-1}). The highest concentration of Ni, 13.72 ± 9.99 mg kg^{-1}, was found in crayfish from the Nová Říše Reservoir, while it was not detected in those from Darkovské moře. The highest Cr content was in Boskovice and Landštejn Reservoirs at 3.76 ± 1.57 and 2.49 ± 2.63 mg kg^{-1}, respectively. Hg content was 0.14 ± 0.09 mg kg^{-1} in Boskovice, while it was half that level in Darkovské moře, at 0.07 ± 0.03 mg kg^{-1}.

3.2. Selected Metal Content in Crayfish Abdominal Muscle. There was no significant difference in Zn content of
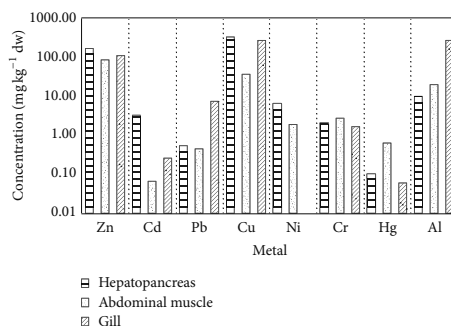

Figure 1: Concentration of metals (zinc, cadmium, lead, copper, nickel, chromium, mercury) in crayfish organ tissues ($n = 10$, for each tissue): hepatopancreas, abdominal muscle, gill. Averages of combined locality data are presented. An ordinate is presented in logarithmic scale.

crayfish abdominal muscle among drinking water reservoirs (Table 9), but approximately double its mean concentration was observed in the contaminated site (128.23 ± 44.33 mg kg^{-1}). Cd content was below the detection limit in drinking water reservoirs but 0.13 ± 0.08 mg kg^{-1} at the contaminated site. Pb was below the detection limit at all sites. The specimens from the contaminated reservoir showed highest Cu content (55.97 ± 14.07 mg kg^{-1}), while the lowest Cu concentration, 20.92 ± 4.34 mg kg^{-1}, was found in Landštejn. No difference was found in either Ni or Cr content among the sampled areas. The highest Hg concentration was seen in Boskovice Reservoir (1.18 ± 0.31 mg kg^{-1}) (Table 9).

3.3. Aluminium Content in Crayfish Gill. Aluminium content of crayfish gill did not significantly differed among water storage reservoirs (50 ± 10–170 ± 130 mg kg^{-1}), while samples from the contaminated locality had significantly higher levels, 780 ± 700 mg kg^{-1} (Table 10).

3.4. Target Tissue of Metal Accumulation in Crayfish. Analysis across all sampling sites indicated that the crayfish digestive organ (hepatopancreas) was the primary accumulation site of the majority of studied metals. This was noted for Zn, Cd, and, to some extent, for Cu and Ni (Figure 1). Cr was found in both hepatopancreas and abdominal muscle but was higher in the latter (Figure 1). Hg primarily accumulated in crayfish abdominal muscle at similar levels for all sites (Tables 8 and 9). Pb and Al mainly accumulated in crayfish gill (Figure 1).

3.5. Reservoir Comparisons. The highest levels of Cd were found in crayfish from Landštejn, while they were the lowest in content of other analysed metals. Darkovské moře showed the highest concentrations of Zn, Pb, Cu, and Al. Nová Říše crayfish had high Zn, Cu, and Ni concentrations. Similar to Darkovské moře and Nová Říše, Boskovice samples showed

TABLE 3: Statistical comparison of metal accumulation in hepatopancreas of crayfish from Boskovice, Landštejn, Nová Říše, and Darkovské moře.

Test	Cd	Cr	Cu	Pb	Hg	Ni	Zn
$KW - H$ (3, 40)	25.81	25.46	32.57	34.39	12.87	5.61[*]	15.54
P	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05	<0.05

[*] df = 2 since nickel was not detected in crayfish hepatopancreas from Darkovské moře.

TABLE 4: Statistical comparison of metal accumulation in abdominal muscle of crayfish from Boskovice, Landštejn, Nová Říše, and Darkovské moře.

Test	Cd	Cr	Cu	Pb	Hg	Ni	Zn
$KW - H$ (3, 40)	33.07	24.36	27.96	39.00	27.35	6.54[*]	23.55
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

[*] df = 2 since nickel was not detected in crayfish abdominal muscle from Darkovské moře.
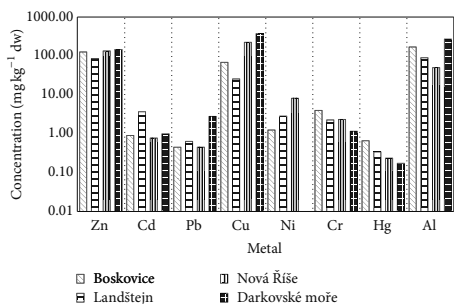

FIGURE 2: Concentration of metals (zinc, cadmium, lead, copper, nickel, chromium, mercury) in crayfish ($n = 10$, for each site) from selected localities (Boskovice, Landštejn, Nová Říše, Darkovské moře). Averages of combined data, abdominal muscle, hepatopancreas, and gill are presented. An ordinate is presented in logarithmic scale.
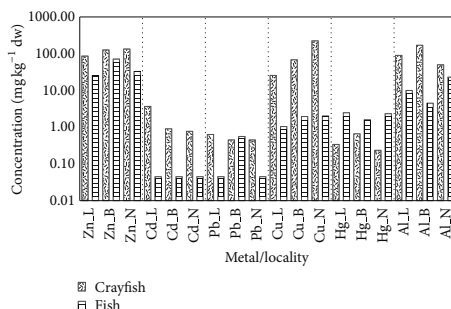

FIGURE 3: Concentration of metals (zinc, cadmium, lead, copper, mercury, and aluminium) in crayfish tissue (abdominal muscle, hepatopancreas, gill) and fish muscle from selected water storage reservoirs: Boskovice (B, crayfish $n = 10$, fish $n = 6$), Landštejn (L, crayfish $n = 10$, fish $n = 5$), Nová Říše (N, crayfish $n = 10$, fish $n = 5$). An ordinate is presented in logarithmic scale.

high Zn content, as well as high Cr and Hg concentrations (Figure 2).

3.6. Selected Metal Content in Fish and Crayfish from Drinking Water Reservoirs. Metal accumulation in crayfish hepatopancreas, abdominal muscle, and gill tissue and in fish muscle tissues (Table 11) was compared among drinking water reservoirs. In general, metal concentrations were significantly higher in crayfish (Figure 3). The only metal occurring in higher amounts in fish muscle compared to crayfish was Hg ($2.10 \pm 1.77 \, \mathrm{mg\,kg^{-1}}$ versus $0.41 \pm 0.42 \, \mathrm{mg\,kg^{-1}}$), while Pb was found in similar amounts ($0.55 \pm 0.24 \, \mathrm{mg\,kg^{-1}}$ for fish and $0.57 \pm 0.21 \, \mathrm{mg\,kg^{-1}}$ for crayfish), although it tended to be higher in crayfish. Fish from the Boskovice Reservoir had the highest Zn content ($71.50 \pm 34.60 \, \mathrm{mg\,kg^{-1}}$) and the lowest Hg concentration ($1.59 \pm 0.53 \, \mathrm{mg\,kg^{-1}}$). Fish from the Landštejn and Nová Říše reservoirs showed similar levels of Zn ($25.42 \pm 9.57 \, \mathrm{mg\,kg^{-1}}$ and $32.90 \pm 5.88 \, \mathrm{mg\,kg^{-1}}$, resp.). Both fish and crayfish from Landštejn contained the lowest amounts of Cu

($1.04 \pm 0.32 \, \mathrm{mg\,kg^{-1}}$ and $25.67 \pm 6.71 \, \mathrm{mg\,kg^{-1}}$, resp.), while the highest Hg concentration in fish ($2.47 \pm 2.73 \, \mathrm{mg\,kg^{-1}}$) was detected at that site. The lowest Al concentration in fish was in Boskovice Reservoir, where levels were below the detection limit.

3.7. Zinc, Cadmium, Lead, Copper, and Mercury in Fish Muscle Compared with Crayfish Abdominal Muscle. Zinc and Cu content was significantly lower in fish muscle compared with crayfish abdominal muscle, while Pb was near the detection limit in both fish and crayfish ($0.55 \pm 0.24 \, \mathrm{mg\,kg^{-1}}$ and $0.50 \, \mathrm{mg\,kg^{-1}}$, resp.), and Cd was below the detection level, in fish. Hg in fish (perch, $4.00 \pm 1.88 \, \mathrm{mg\,kg^{-1}}$, > pikeperch, $2.33 \, \mathrm{mg\,kg^{-1}}$, > rudd, $2.11 \pm 0.30 \, \mathrm{mg\,kg^{-1}}$, > tench, $1.15 \pm 0.65 \, \mathrm{mg\,kg^{-1}}$, > roach, $0.83 \pm 0.23 \, \mathrm{mg\,kg^{-1}}$, > bream, $0.62 \pm 0.68 \, \mathrm{mg\,kg^{-1}}$) was detected in higher amounts than in crayfish, $0.72 \pm 0.40 \, \mathrm{mg\,kg^{-1}}$, (Figure 4).

TABLE 5: Statistical comparison of metal accumulation in crayfish hepatopancreas and abdominal muscle among drinking water reservoirs.

Locality/Test	Cd	Cr	Cu	Pb	Hg	Ni	Zn
Boskovice							
W-Z (1, 10)	2.70	2.60	2.29	2.80	2.80	1.75	2.80
P	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05	<0.05
Landštejn							
W-Z (1, 10)	2.80	0.06	0.66	2.37	2.80	2.31	2.29
P	<0.05	>0.05	>0.05	<0.05	<0.05	<0.05	<0.05
Nová Říše							
W-Z (1, 10)	2.80	2.70	2.80	2.80	2.80	2.50	2.80
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

TABLE 6: Statistical comparison of metal accumulation in crayfish hepatopancreas, abdominal muscle, and gill for Darkovské moře Reservoir.

Test	Al	Cd	Cr	Cu	Pb	Hg	Zn
F (2, 10)	16.00	15.62	10.57	20.00	20.00	15.44	16.80
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

TABLE 7: Statistical evaluation of aluminium accumulation in gill of crayfish from Boskovice, Landštejn, Nová Říše, and Darkovské moře.

Test	Al
KW − H (3, 25)	18.68
P	<0.05

n = 25 since gill of crayfish from Boskovice, Landštejn, and Nová Říše were pooled into 5 samples for each locality.

4. Discussion

As expected, hepatopancreas of crayfish showed the highest accumulation rate for the majority of evaluated metals. Thus, when the goal is to obtain relevant content of Zn, Cd, Cu, or Ni in crayfish as biomonitors it is advisable to assess levels in hepatopancreas. Analysing other tissues for these metals may result in concentrations appearing low or remaining undetected.

Cr can be detected in hepatopancreas and abdominal muscle in relatively equal amounts. Although Cr is toxic to aquatic organisms [12], it is not included as potentially hazardous to humans by the European Commission (EC) Regulation [13] setting maximum levels for foods. Jorhem et al. [11] reported that Cr concentrations in hepatopancreas of the noble crayfish rose several-fold after boiling. Jorhem et al. [11] and Mackevičienė [14] found Cr concentrations in abdominal muscle of noble crayfish caught in unpolluted Swedish and Lithuanian lakes to be 0.13 and 0.30 mg kg^{-1}, respectively, and in hepatopancreas, 0.15 and 0.25 mg kg^{-1}, respectively, compared to our findings of 2.03–4.19 mg kg^{-1} for abdominal muscle and 0.87–3.76 mg kg^{-1} for hepatopancreas. The lowest concentrations of Cr were found in Darkovské moře, which was regarded a contaminated site. Tunca et al. [15], reported similar Cr concentrations in hepatopancreas (0.65 mg kg^{-1}) and abdomen (0.50 mg kg^{-1}) of the narrow-clawed crayfish from a Turkish lake, collected in the same season as our
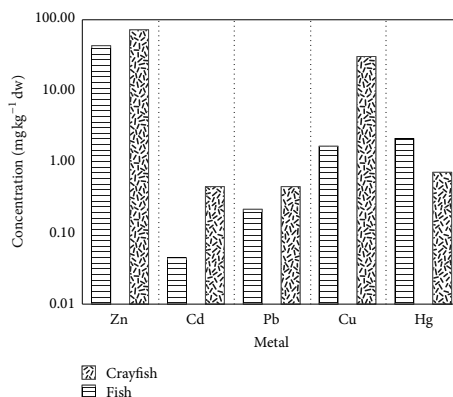

FIGURE 4: Concentration of selected metals (zinc, cadmium, lead, copper, mercury) in crayfish (*n* = 30) abdominal muscle and fish (*n* = 16) muscle throughout water reservoirs (means of combined data Boskovice, Landštejn, Nová Říše). An ordinate is presented in logarithmic scale.

study, at an unpolluted site. Tunca et al. [16] reported Cr concentration in gill to be somewhat higher than in our study (5.3 mg kg^{-1} versus 1.67 mg kg^{-1}), as was Ni, which was not detected in the crayfish from Darkovské moře. Although Tunca et al. [16] found a positive Cr/Cu correlation (*r* = 0.53) that could explain some trends of Cr accumulation in crayfish tissue, we found no relationship between these metals in our survey.

Zn, Cu, and Ni, which commonly accumulate mainly in hepatopancreas of other crayfish species as well [7], were found to be substantially decreasing after cooking [11]. However, concentrations of Zn and Cu in abdominal

TABLE 8: Metal concentration (mg kg^{-1} dw) in crayfish (n = 10 from each locality) hepatopancreas. Values are given as mean ± s.d.

Locality	Aluminium	Cadmium	Chromium	Copper	Lead	Mercury	Nickel	Zinc
Boskovice	N/D	1.76 ± 0.43[b]	3.76 ± 1.57[a]	103.97 ± 105.37[bc]	<0.50[b]	0.14 ± 0.09[a]	1.11 ± 1.33[b]	176.10 ± 56.22[a]
Landštejn	N/D	7.31 ± 2.56[a]	2.49 ± 2.63[ab]	30.41 ± 32.22[c]	0.82 ± 0.35[a]	0.10 ± 0.03[ab]	3.89 ± 2.60[ab]	100.29 ± 34.98[b]
Nová Říše	N/D	1.50 ± 0.40[b]	0.87 ± 1.08[b]	410.10 ± 154.70[ab]	<0.50[b]	0.08 ± 0.04[ab]	13.72 ± 9.99[a]	199.60 ± 59.80[a]
Darkovské moře	10 ± 10	2.58 ± 1.36[b]	0.80 ± 0.67[b]	794.70 ± 234.74[a]	<0.50[b]	0.07 ± 0.03[b]	N/D	200.10 ± 58.46[a]

[a,b,c] Values marked by different letters differed significantly at $\alpha < 0.05$.
N/D Metal was not detected.

TABLE 9: Metal concentration (mg kg^{-1} dw) in crayfish (n = 10 from each locality) abdominal muscle. Values are given as mean ± s.d.

Locality	Aluminium	Cadmium	Chromium	Copper	Lead	Mercury	Nickel	Zinc
Boskovice	N/D	<0.05[b]	4.19 ± 6.82[a]	32.89 ± 7.94[bc]	<0.50[a]	1.18 ± 0.31[a]	1.46 ± 1.38[a]	76.87 ± 11.01[b]
Landštejn	N/D	<0.05[b]	2.03 ± 1.00[a]	20.92 ± 4.34[c]	<0.50[a]	0.58 ± 0.06[ab]	1.77 ± 3.40[a]	71.98 ± 7.98[b]
Nová Říše	N/D	<0.05[b]	3.79 ± 5.94[a]	37.60 ± 9.00[ab]	<0.50[a]	0.39 ± 0.15[b]	2.58 ± 6.24[a]	68.60 ± 4.57[b]
Darkovské moře	20 ± 30	0.13 ± 0.08[a]	0.99 ± 0.84[a]	55.97 ± 14.07[a]	<0.50[a]	0.39 ± 0.16[b]	N/D	128.23 ± 44.33[a]

[a,b,c] Values marked by different letters differed significantly at $\alpha < 0.05$.
N/D Metal was not detected.

muscle have been shown to slightly increase after boiling [11]. Zn, Ni, and Cu are also not considered potentially harmful to humans according to EC regulations [13]. From an animal welfare point of view, Zn and Ni concentrations in our survey were in agreement with reviews of Eisler [17, 18] and results of Jorhem et al. [11] and Mackevičienė [14], who reported Ni levels (0.50–0.85 mg kg^{-1}) similar to our observations (1.38–2.55 mg kg^{-1}) in abdominal muscle and 1.54–3.54 mg kg^{-1} compared with our findings (1.11–13.72 mg kg^{-1}) in hepatopancreas. Zinc and Cu in abdominal muscle (23.25–75.00 mg kg^{-1} versus 68.60–128.23 mg kg^{-1} and 6.10–28.50 mg kg^{-1} versus 20.92–55.97 mg kg^{-1}, resp.) and Cu in hepatopancreas (4.93–185.71 mg kg^{-1} versus 30.41–794.70 mg kg^{-1}) were found in higher concentrations in specimens from the Czech reservoirs than in those from Swedish and Lithuanian waters [11, 14]. Reported differences can largely depend on differing geological characteristics of localities, including environmental concentrations of metals in reservoirs. The highest concentrations of these metals in our study were found in the contaminated site, but not in drinking water basins. This may be linked to mining activity near the contaminated location. In agreement with Bagatto and Alikhan [19, 20], we also observed hepatopancreas to be the major crayfish organ for metal accumulation. However, Tunca et al. [15] reported Cr, Ni, and Cu to accumulate in crayfish gill at greater levels than in hepatopancreas.

When metal content was averaged for all tested crayfish tissues and compared with those in fish, we found no obvious similarities in levels or distribution. In general, metal concentrations were higher in crayfish than in fish. When comparing crayfish abdominal muscle to that of fish muscle, we did not find relationships among concentrations of metals. The majority of analysed elements (Zn, Cd, Pb, and Cu) appeared at higher concentrations in crayfish than in fish (Figure 4). The only element with higher concentration in fish than in crayfish was Hg. Fish data in this study included species feeding on benthic invertebrates (tench), on plankton

or benthic invertebrates (roach, bream), and on zooplankton or algae (rudd), as well as predatory fish (pikeperch and perch). It is not surprising that the highest Hg concentrations were found in pikeperch, 3.54–6.13 mg kg^{-1}, and perch, 1.06–2.33 mg kg^{-1}, since these species occupy a higher trophic level [21]. Since Hg biomagnifies through the food web [22, 23], Hg content in fish would be expected to exceed that in crayfish. If crayfish prey upon other benthic invertebrates, Hg biomagnification would be a factor in those species also [24].

The highest Hg levels were found in crayfish from the Boskovice Reservoir, which did not appear to be the most contaminated among the studied sites with respect to other metals. We cannot suggest that Boskovice is the site polluted by Hg because of its highest amounts there, as this metal is actively transported through the trophic web [25], particularly in the initial years of reservoir exploitation. In this connection, Boskovice Reservoir was the youngest, constructed in 1990, while Nová Říše and Landštejn reservoirs have been in use since 1985 and 1973, respectively. There is no information on Hg content in crayfish abdominal muscle in EC Regulations [13], but 1.00 mg kg^{-1} of Hg in muscle of fresh fish is within safe limits for the human health and for aquatic animal welfare [26]. Our data for the omnivorous fish, bream and roach, are in agreement with Noël et al. [8], who looked at fish from uncontaminated sites, but Hg content for predatory perch (0.47 mg kg^{-1}) and pikeperch (0.94 mg kg^{-1}) was several times higher in our study (4.00 mg kg^{-1} and 2.33 mg kg^{-1}, resp.). However, we analysed larger fish, perch of average 434 g compared to 105 g and pikeperch of average 3300 g compared to 1002 g. Červenka et al. [27], who analysed fish muscle from fresh water reservoirs, observed similarly high, 6.41 mg kg^{-1}, Hg levels in perch, but higher levels in bream, 2.78 mg kg^{-1} compared with 0.64 mg kg^{-1} in the present study. Svobodova et al. [28] found less than 0.05 mg kg^{-1} (fresh weight) Hg in common carp *Cyprinus carpio* (L. 1758); however, with respect to Cd, Pb, and Cu our results are in agreement. Cd and Cu in

TABLE 10: Aluminium concentration ($mg\,kg^{-1}$ dw) in crayfish ($n = 10$ from each locality) gill. Values are given as mean ± s.d.

Locality	Boskovice	Landštejn	Nová Říše	Darkovské moře
Aluminium	170 ± 130^{ab}	90 ± 50^{b}	50 ± 10^{b}	780 ± 700^{a}

[a,b] Values marked by different letters differed significantly at $\alpha < 0.05$.

TABLE 11: Metal concentration ($mg\,kg^{-1}$ dw) in muscle of fish ($n = 16$) from drinking water reservoirs.

Reservoir/Species	Aluminium	Cadmium	Copper	Lead	Mercury	Zinc
Boskovice						
Abramis brama	<5.0	<0.05	1.28	<0.5	1.41	33.3
Scardinius erythrophthalmus	<5.0	<0.05	2.95	<0.5	2.32	126.0
Scardinius erythrophthalmus	<5.0	<0.05	1.55	<0.5	1.90	69.8
Perca fluviatilis	<5.0	<0.05	1.55	0.62	1.06	97.3
Rutilus rutilus	<5.0	<0.05	1.92	<0.5	0.99	45.3
Tinca tinca	<5.0	<0.05	2.42	0.51	1.88	57.3
Landštejn						
Abramis brama	6.00	<0.05	<0.5	1.01	0.24	41.8
Perca fluviatilis	16.00	<0.05	<0.5	1.16	4.66	23.9
Perca fluviatilis	11.00	<0.05	0.93	0.93	6.13	16.9
Rutilus rutilus	7.00	<0.05	<0.5	1.50	0.66	23.5
Tinca tinca	<5.00	<0.05	<0.5	0.62	0.64	21.0
Nová Říše						
Abramis brama	<5.0	<0.05	<0.5	1.34	0.21	38.5
Perca fluviatilis	<5.0	<0.05	<0.5	5.33	3.57	32.7
Perca fluviatilis	<5.0	<0.05	<0.5	1.03	4.59	38.8
Sander lucioperca	<5.0	<0.05	<0.5	0.66	2.33	25.2
Tinca tinca	23.00	<0.05	1.33	1.69	0.93	29.3

fish also did not exceed values established by EC Regulations [13] and Eisler [29, 30]. The noble crayfish is regarded as an Hg bioindicator by Loukola-Ruskeeniemi et al. [31], who detected concentrations in crayfish abdominal muscle ($0.88\,mg\,kg^{-1}$) similar to that of the present study (0.39–$1.18\,mg\,kg^{-1}$) in Finland, while we found less Hg in hepatopancreas, 0.07–$0.14\,mg\,kg^{-1}$, compared with $0.24\,mg\,kg^{-1}$ reported for Finnish crayfish.

We analysed crayfish gill for presence of Al, as its concentration could serve as a reference value for investigation of impact on crayfish of aluminium-containing compounds employed for water treatment. The common coagulation agent PAX-18 is such a compound, as it contains polyaluminium chloride (9% aluminium) as an active ingredient and is widely used to precipitate orthophosphates, which cause water eutrophication, and to reduce phytoplankton bloom [32]. However, together with phytoplankton control, polyaluminium chloride can cause harmful effects in nontarget aquatic organisms, especially those most vulnerable to the impact and that easily accumulate aluminium, primarily juvenile fish [32] and crustaceans [33], but also other benthic organisms.

It is difficult to assess the level of toxicity of the observed aluminium, and whether the levels found in crayfish gill are the result of metal pollution. The high, $780\,mg\,kg^{-1}$, aluminium level in gill of crayfish from the contaminated site presumes its contamination. Similar Al levels were reported

by Madigosky et al. [34], who found up to $981\,mg\,kg^{-1}$ in gill of the red swamp crayfish, *Procambarus clarkii* (Girard, 1852), from road-side ditches along highways in northern Louisiana, USA. While we primarily focused on drinking water reservoirs, it is necessary to consider Darkovské moře. Al concentrations in hepatopancreas at $10\,mg\,kg^{-1}$ and abdominal muscle at $20\,mg\,kg^{-1}$ were found in crayfish from the site. Alexopoulos et al. [35], after 20-day exposure to Al at $500\,\mu g\,L^{-1}$, found approximately $1200\,mg\,kg^{-1}$ in signal crayfish *Pacifastacus leniusculus* (Dana, 1852) gill, $10\,mg\,kg^{-1}$ in abdominal muscle, and $20\,mg\,kg^{-1}$ in the digestive gland. Gill concentration measured in our study would correspond approximately to 14 days of such exposure, while in abdominal muscle and hepatopancreas, Al levels would be half that value, but still of a similar effect. According to Macova et al. [32], amounts of PAX-18 commonly used for treatment of natural waters, 45–$90\,mg\,kg^{-1}$ equivalent to 5–$10\,mg\,kg^{-1}$ of Al, are safe for common carp juveniles. The treatment dose in Alexopoulos et al. [35] was 10% of that commonly used. Therefore, we can suppose that Al content in crayfish gill from the Czech drinking water reservoirs, 50–$170\,mg\,kg^{-1}$, was not evidence of contamination, as these concentrations were much lower than those found in contaminated site. As in crayfish, gill is the target for Al uptake in fish [36, 37], since Al binds to the gill due to the mucus secreted by these organs that causes their damage and mucus intensive buildup [38, 39].

Fish muscle showed lower, $4.5–23.0 \, \text{mg kg}^{-1}$, Al concentrations compared to those reported by Coetzee et al. [40], 11–$109 \, \text{mg kg}^{-1}$, and $22.5–40.6 \, \text{mg kg}^{-1}$ found by Sapozhnikova et al. [41]. Although the highest Al concentrations are usually accumulated in gill, we agree with Coetzee et al. [40] that, in monitoring, muscle should also be considered.

5. Conclusions

Various aquatic organisms should be used in biomonitoring studies to give a more complete picture of environmental pollution. Crayfish, due to low migration across water bodies, may provide more precise data than do fish. In biomonitoring, some potentially toxic elements, such as Al and Hg, can be over- or underestimated, depending on considered tissue and species and the stage of their life cycle. Thus either crayfish abdominal or fish muscle for Al bioaccumulation assessment is recommended, while for Hg surveys, fish, especially carnivorous species, should not be used because of their potential for biomagnification. The remaining metals, Cd, Cu, Ni, and Zn, except of Cr and Pb, are primarily accumulated in crayfish hepatopancreas, making this tissue the recommended target for bioaccumulation studies. Expansion of data sources, species, tissues, and sampling sites will produce more relevant biomonitoring surveys. The last is highly important, not just for environmental preservation, but for evaluation of the potential effects on human health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Stress reaction in crayfish: chlorides help to withstand stress in high nitrite concentration conditions – preliminary study

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ABSTRACT

Key-words:
Astacus
leptodactylus,
cardiac activity,
heart rate,
salinity,
stress reaction

A non-invasive method of recording cardiac activity (heart rate – HR) and stress reaction (stress index – SI) was used to understand the immediate and ongoing stress reaction of crayfish to the chemical stimuli. This method detects changes in the shape and amplitude parameters of the response to the stress factors, which characterized the crayfish functional state. Experimental animals (*Astacus leptodactylus*) were divided to the two groups with (400 mg·L^{-1} Cl$^-$) and without added chlorides and then exposed to a stepwise increased level of nitrite to the final (sublethal-lethal) concentration of 60 mg·L^{-1} N-NO$_2^-$ within 24 hours. The course of crayfish reaction was evident and provided information about their reaction to the sublethal-lethal concentration over time. As expected, a less prominent stress reaction was detected in the group with chlorides. The non-invasive method successfully evaluated the sensing of chemical stimuli in water through HR and SI changes.

RÉSUMÉ

Réaction au stress chez l'écrevisse : les chlorures aident à résister au stress dans des conditions de fortes concentrations en nitrite – étude préliminaire

Mots-clés :
Astacus
leptodactylus,
activité
cardiaque,
rythme
cardiaque,
salinité,
réaction
au stress

Une méthode non-invasive d'enregistrement de l'activité cardiaque (rythme cardiaque – HR) et de réaction au stress (indice de stress – SI) a été utilisée pour comprendre la réaction au stress immédiate et ultérieure de l'écrevisse à des stimuli chimiques. Cette méthode détecte les changements dans la forme et l'amplitude des paramètres de la réponse aux facteurs de stress, qui caractérisent l'état fonctionnel de l'écrevisse. Les animaux de l'expérience (*Astacus leptodactylus*) ont été divisés en deux groupes avec (400 mg Cl$^-$·L^{-1}) et sans chlorures ajoutés puis exposés à des niveaux croissants par paliers de nitrite jusqu'à la concentration finale (sub-létale–létale) de 60 mg·L^{-1} N-NO$_2^-$ pendant 24 heures. La réaction de l'écrevisse est évidente et fournit des informations sur les réactions au cours du temps à une concentration sub-létale. Comme escompté, une réaction au stress moins marquée est détectée dans le groupe avec des chlorures. La méthode non-invasive a déterminé correctement l'impact du stimulus chimique de l'eau par les changements de HR et SI.

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INTRODUCTION

Toxicity tests usually describe a direct impact of several chemicals on model organisms. The results then express the concentration of a given chemical which caused mortality of tested organisms in a limited time period (Buikema *et al.*, 1982). Nevertheless, much lower (sublethal) concentrations of a chemical could influence the physiological and functional state or behaviour of treated organisms (Kleerekoper, 1976; Schober and Lampert, 1977; Vernberg *et al.*, 1977). Sublethal poisoning may be detectable after a longer time period or even be unnoticeable. Moreover, it may be difficult to detect stress reactions in such cases. Several invasive and non-invasive techniques could be used to evaluate the physiological state of fish (*e.g.* Kane *et al.*, 2004, 2005) and crayfish (*e.g.* Bierbower and Cooper, 2009). Both of techniques give accordingly good results. Invasive monitoring is based on method of electrocardiography and uses crayfish as measuring device including animal directly to the measuring circuit (Bierbower and Cooper, 2009). But transduction of physiological data involved invasive procedures such as electrode implantation, which increase physiological disturbance such as unneeded rising of heart rate (Aagaard *et al.*, 1991). One of the non-invasive methods (used in our study) based on the evaluation of the physiological state by measurements of heart rate and cardiac activity patterns was developed for such purposes by Kholodkevich *et al.* (2007). Heart rate and cardiac activity patterns are the key indicators of the condition of tested organisms and can be expressed as stress dependent factors. These measurements are included in all general clinical assessments in medical science (Swash and Mason, 1984) and are therefore used also for the stress index calculation (Kholodkevich *et al.*, 2008). At present, data regarding fish or crayfish stress reactions to sublethal-lethal levels of poisoning are often lacking. The non-invasive method of detection of heart rate and stress reaction could open a new window of knowledge in toxicology and biomonitoring. It is supposed that crayfish are capable to react quickly to any chemical changes by a stress reaction rather than toxicity thresholds resulting in death of specimens. Numerous authors have found heart rate to be a useful indicator of changes in physiological state, even in crustacean, molluscs and fish (*e.g.* Depledge and Andersen, 1990; Kholodkevich *et al.*, 2008).

Nitrites (NO_2^-) are usually found together with nitrates and ammonia in water. Ammonia (NH_3) is considered one of the most important contaminants in aquatic environment because of its toxic nature and ubiquity in surface water system. It is discharged into the environment in large quantities from industrial, municipal and agricultural sources (Bloxham *et al.*, 1999). In aquatic ecosystems, nitrite concentrations are elevated by pollution with nitrogenous wastes and imbalances in bacterial nitrification and denitrification processes (Eddy and Williams, 1987). The average concentration of $N-NO_2^-$ in ground water ranges from 0.004 to 0.179 mg·L^{-1}. Higher concentrations (more than 1 mg·L^{-1}) occur in wastewater (Pitter, 1999) and in intensive aquaculture recirculating systems usually in newly activated biofilters (Masser *et al.*, 1999). In fish, nitrites are absorbed by the gills and oxidize the iron in the haemoglobin molecules to methemoglobin. The result of nitrite poisoning is methaemoglobinemia caused by a reduction in the blood oxygen carrying ability (Svobodová *et al.*, 2005). It is likely that a similar type of reaction to that occurring with the iron of haemoglobin also occurs with the copper in crustacean hemocyanin (Colt and Armstrong, 1981). Nitrites have an affinity for the active chloride uptake mechanism by chloride cells in the gills. Chloride cells excrete ammonia or H^+ ions for Na^+ and bicarbonate (HCO_3^-) for Cl^- (Love, 1980). Nitrites thus have an affinity to Cl^-/HCO_3^- exchange. Part of chloride demand is replaced by NO_2^- when available in water. The higher concentration of chlorides therefore protects fish against the toxic impact of nitrite *via* the competition between chloride and nitrite ions transporting across the gill membrane (Jensen, 2003). The positive effect of chloride on tolerance to nitrites has been demonstrated in other studies in fish (Hilmy *et al.*, 1987; Atwood *et al.*, 2001; Huertas *et al.*, 2002; Fuller *et al.*, 2003; Taveres and Boyd, 2003) and crayfish (Beitinger and Huey, 1981; Jeberg and Jensen, 1994; Kozák *et al.*, 2005).

This study was concerned with 1) a preliminary testing of the reactivity of crayfish to the common chemical, nitrite, to gain information about sensitiveness to an increasing level of chemical stimuli up to the sublethal-lethal concentration and 2) to evaluate if chlorides could

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help crayfish to withstand stress in high nitrite concentration conditions. The sensitivity of a non-invasive method for heart rate and stress index monitoring was also evaluated. This study can offer some suggestions and recommendations for future research of heart rate and stress monitoring in crayfish or fish.

MATERIAL AND METHODS

The experiment was conducted in the Laboratory of Experimental Ecology of Aquatic Systems, Saint-Petersburg Scientific Research Center for Ecological Safety, Russia.

> EQUIPMENT

A fibre-optic method originally developed for recording cardiac activity of Crustacea (Decapoda) and Mollusca was used (Fedotov et al., 2000; Kholodkevich et al., 2008). The optical signal modulated by the heart of the tested animal contains information on cardiac activity which is processed by a personal computer (for more details see Kholodkevich et al., 2008) (Figure 1). The results create a photoplethysmogram, which can be further analyzed by various mathematical and statistical methods. The variation pulsometry method was used to study the distribution of cardiac intervals, and analyze relationships between its shape and the functioning of the cardiac system (Kholodkevich et al., 2008) (Figure 2). For this method the following characteristics were chosen as biomarkers: heart rate (HR), and stress-index (SI), which is defined by the formula:

$$SI = 1/(2 \, {}^{*}CI_m {}^{*}SD^2),$$

where CI_m = mean cardiac interval, which is related to HR, with HR = $60/CI_m$; SD = standard deviation of heart rate (Kholodkevich et al., 2007, 2008).

> ANIMALS AND EXPERIMENTAL CONDITIONS

Narrow-clawed crayfish originating from Sevan Lake (Armenia) were used in the experiment. The animals were acclimatized to the controlled conditions for three weeks before the experiment. They were kept in a trough ($2.5 \times 0.5 \times 0.3$ m) with shelters in a recirculating system. During the experiment, each crayfish was maintained in a separate 5-liter aquarium with a shelter, which allowed the crayfish to stay inside (Figure 3). The water level was set at 15 cm. Crayfish were not fed during the experiment. The non-treated water quality parameters were as follows: temperature 22 °C, oxygen saturation 80–100%, pH 7.5, HCO_3^- 32.5 mg·L^{-1}, SO_4^{2-} 19.3 mg·L^{-1}, Cl^- 7.8 mg·L^{-1}, Ca^{2+} 11.3 mg·L^{-1}, Mg^{2+} 2.9 mg·L^{-1}, Na^{2+} 3.3 mg·L^{-1}, K^+ 1.5 mg·L^{-1}. The experiment was performed at the September photoperiod (D:N = 14:10).

> EXPERIMENTAL DESIGN

The study was conducted in two groups, with added chlorides (Cl^-; 400 mg·L^{-1}) and without chlorides. The chloride level of 400 mg·L^{-1} was chosen as relatively safe for crayfish (Kozák et al., 2009) and helpful against a negative impact of nitrites (Kozák et al., 2005). Each group consisted of three adult males, with total length (TL) = 131.3 ± 3.5 mm, carapace length (CL) = 70.7 ± 2.2 mm and weight (w) = 63.7 ± 4.1 g in the chlorides group, and TL = 130.7 ± 3.9 mm; CL = 71.0 ± 2.6 mm and w = 70.3 ± 5.5 g in the group without chlorides, respectively. One monitoring system at full capacity monitored all six treated specimens during the 24 hours period of treatment.

Animals were stocked in aquaria for acclimation in the evening before the day of the experiment. The concentration of 60 mg N-NO_2^-·L^{-1} was chosen as a possible sublethal-lethal
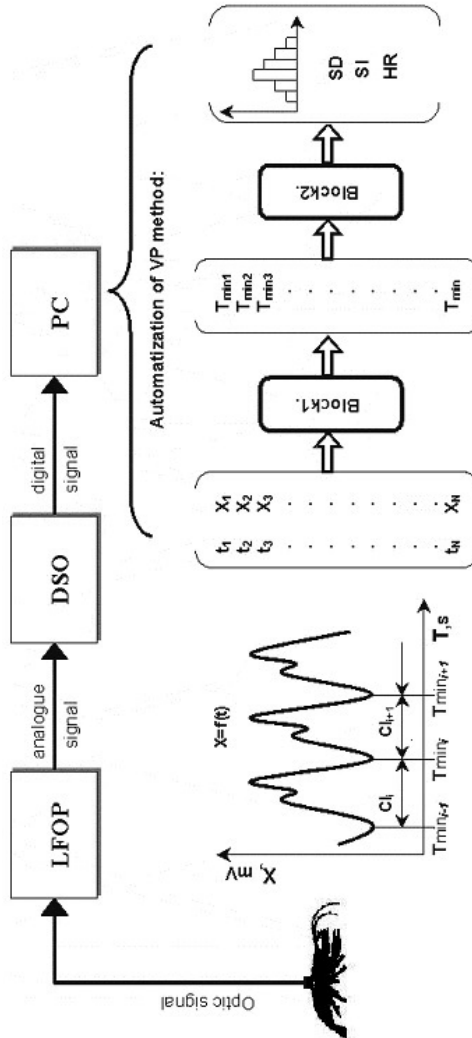
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Figure 1
Block-scheme of cardiac activity registration, signal transformation and automatic data processing in real time. LFOP: laser fibre-optic photoplethysmograph, DSO: digital storing oscilloscope, PC: personal computer, VP: variation pulsometry, SD: standard deviation, HR: heart rate, SI: stress-index (Kholodkevich et al., 2008).

Figure 1
Bloc-diagramme de l'enregistrement de l'activité cardiaque, transformation du signal et traitement en temps réel des données. LFOP : photopléthysmographe à fibre optique laser, DSO : oscilloscope à mémoire, PC : ordinateur personnel, VP : variation de pulsations, SD : déviation standard, HR : rythme cardiaque, SI : indice de stress (Kholodkevich *et al.*, 2008).
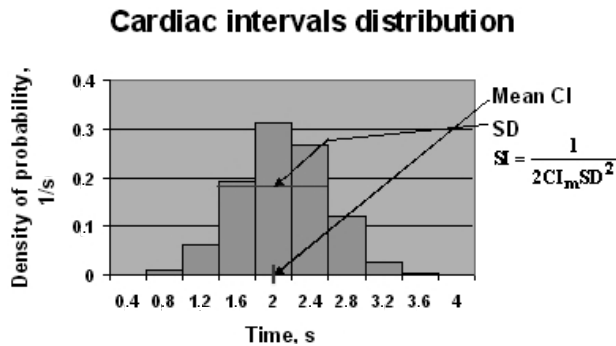
Figure 2
Variation pulsometry method to study relations between cardiac system functioning and the cardiac intervals distribution law (Kholodkevich et al.*, 2008).*

Figure 2
Méthode de variation de pulsations pour étudier les relations entre le fonctionnement cardiaque et la loi de distribution des phases d'activité cardiaque (Kholodkevich *et al.*, 2008).
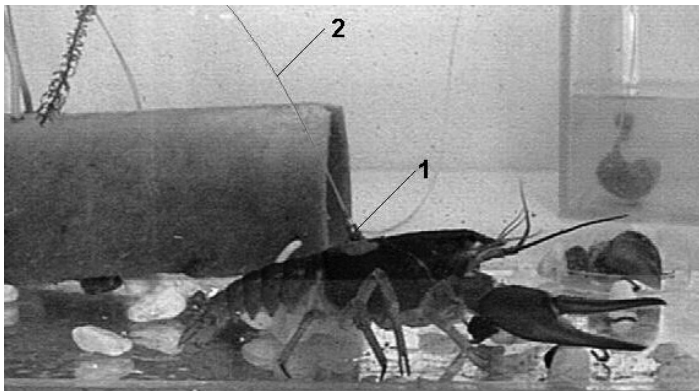

Figure 3
*Narrow-clawed crayfish (*Astacus leptodactylus*) in 5-L aquarium with a shelter, which allowed the crayfish with sensor (1) and fibre (2) attached to stay inside (Kholodkevich* et al.*, 2008).*

Figure 3
Écrevisse à pattes grêles (*Astacus leptodactylus*) dans un aquarium de 5 L avec un abri qui accueille l'écrevisse équipée de senseur (1) et fibre (2) de (Kholodkevich *et al.*, 2008).

concentration for crayfish in the short time exposure according to the literature and previous experiments with *Orconectes limosus* (Kozák *et al*., 2005). The relevant amounts of Cl⁻, dissolved in 100 mL of water and the first concentration of nitrite (3.75 mg $N\text{-}NO_2^-\cdot L^{-1}$), were applied together to aquaria. Nitrite concentrations were increased every two hours from the initial level of 3.75 mg $N\text{-}NO_2^-\cdot L^{-1}$ through concentrations of 7.5, 15, 30 mg $N\text{-}NO_2^-\cdot L^{-1}$, to the final concentration of 60 mg $N\text{-}NO_2^-\cdot L^{-1}$. Crayfish exposure in each nitrite concentration therefore lasted for two hours. Crayfish were then exposed to the highest concentration for 16 hours. Crayfish were then placed in fresh water without any additional chlorides or nitrites for one day. Samples of water for chemical analysis were taken regularly from each concentration.

Figure 4
The hanging of crayfish – crayfish is hanged on wire above the floor, provided to evaluate their physiological stage.

Figure 4
Écrevisse suspendue au-dessus du fond pour évaluer son état physiologique.

> DATA ANALYSIS

Data were edited and examined using ANOVA for repeated measurements with Tukey *post-hoc* test in program Statistica 9.0 (StatSoft., Inc.). Data are presented as means ± SE.

RESULTS AND DISCUSSION

We demonstrated the effectiveness of the non-invasive method for monitoring cardiac activity of crayfish exposed to the sublethal-lethal nitrite concentration. Crayfish reaction to excess nitrite was detectable (Figure 5). It can be presumed that a similar type of reaction as known in fish, *i.e.* accumulation of nitrites resulting in subsequent methaemoglobinemia (Kroupová *et al*., 2005; Svobodová *et al*., 2005), occurs in copper of crustacean hemocyanin (Colt and Armstrong, 1981; Rainbow, 2002; Kouba *et al*., 2010). We found a difference in both heart rate and stress index and the "chloride" group could be distinguished on the basis of obtained data. The stress index (SI) was already found to be elevated from the 2nd tested N-NO$_2^-$ level (7.5 mg·L^{-1}) in the group without chlorides. Reactions in crayfish to the treatment with increased chlorides were observed from the N-NO$_2^-$ level of 30 mg·L^{-1}. The course of reactions to increasing nitrite levels, in both SI and HR, was significantly influenced by the presence of chlorides. Heart rates of animals without added salt presented fluctuating values independently of nitrite exposure as a probable impact of their toxicity. Regarding heart rate, the loss of its circadian rhythmicity has been presented as a potential early warning indicator for mortality in freshwater crab *Potamon potamios* and noble crayfish (*Astacus astacus*) after toxication (Styrishave and Depledge, 1996).

Generally, crayfish in the group without chlorides showed more distinct reactions. Such status is presumably related to maintaining homeostasis in both crayfish and fish (Maetz, 1971; Wheatly and Gannon, 1995), and the related competition of chloride and nitrite ions in the gill chloride cells. Crayfish in both groups were insensible and "near to death" at the end of observations, but with different stress reactions (Figures 4, 6–8). Such harmful effects are not surprising when we consider that values of 96hLC50 for N-NO$_2^-$ usually range from 0.66

05p6

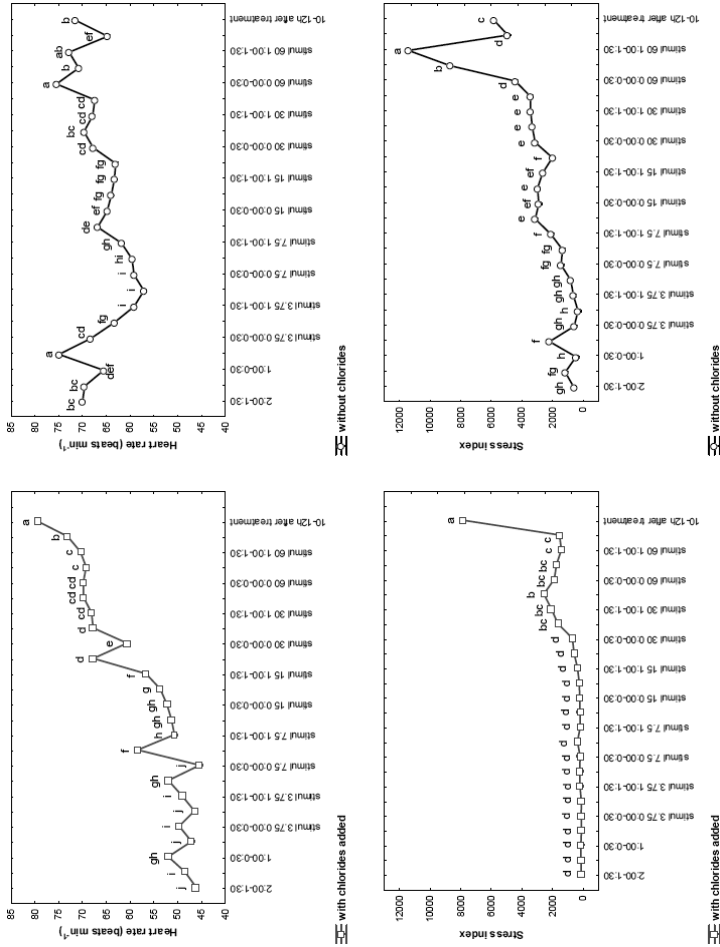P. Kozák et al.: Knowl. Managt. Aquatic Ecosyst. (2011) 401, 05

Figure 5
The course of crayfish reaction (heart rate, stress index) at different nitrite levels during experiments. Right column represents group with (400 mg $Cl^- \cdot L^{-1}$) and left column shows data for the group without an addition of chlorides. Data are presented as mean ± SE. Different superscripts differ at α = 0.05 (ANOVA, F = 400.1, P < 10^{-6}).

Figure 5
Déroulement de la réaction de l'écrevisse (rythme cardiaque, indice de stress) à différentes concentrations de nitrite pendant les expériences. La colonne de droite représente le groupe dans l'eau avec chlorures (400 mg $Cl^- \cdot L^{-1}$) et celle de gauche le groupe sans addition de chlorures. Les données sont des moyennes avec écart-type. Les différentes lettres diffèrent au niveau α = 0,05 (ANOVA, F = 400,1 ; P < 10^{-6}).

05p7

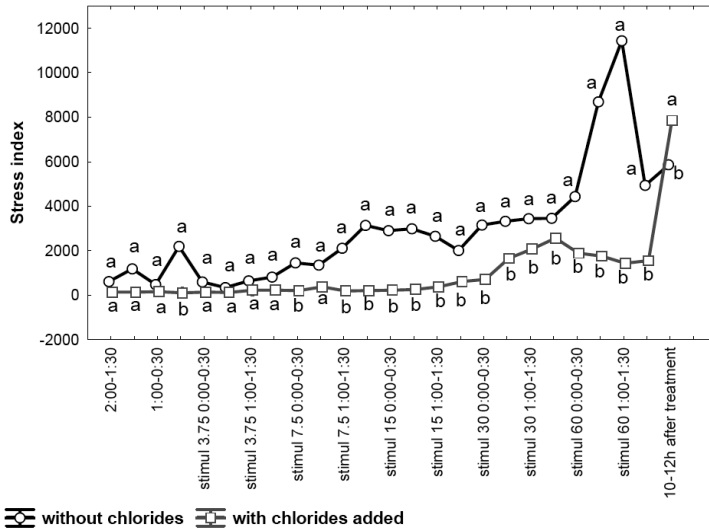**Figure 6**
The comparison of crayfish stress index at different nitrite levels between group with (400 mg Cl⁻·L⁻¹)
and without an addition of chlorides. Data are presented as mean ± SE. Different superscripts differ at
$\alpha = 0.05$ *(ANOVA, F = 238.7, P < 10⁻⁶).*

Figure 6
Comparaison des indices de stress de l'écrevisse soumise à différentes concentrations de nitrite entre le
groupe dans l'eau avec chlorures (400 mg Cl⁻·L⁻¹) et celui sans addition de chlorures. Les données sont
des moyennes avec écart-type. Les différentes lettres diffèrent au niveau $\alpha = 0,05$ (ANOVA, *F* = 238,7 ;
P < 10⁻⁶).

to 200 mg·L⁻¹ in freshwater fish, or commonly between 8.5 and 15.4 mg·L⁻¹ for crustaceans
(Boyd, 1990 in Rouse *et al*., 1995). In general, the N-NO₂⁻ concentration of 60 mg·L⁻¹ was
critical for both groups. However, crayfish were placed into fresh water for 24 hours following
nitrite exposure. While all crayfish from the group without chlorides died within this time, the
other group was capable of fast recovery under fresh water conditions and survived. Chloride
presence therefore enabled crayfish to withstand and survive very high nitrite concentration.

On the other hand, toxic chemicals such as nitrite have visible negative effects when ex-
ceeded – the danger of direct death of animals. However, sublethal effects of nitrites or other
chemicals should not be underrated. It is known that elevated concentrations of nitrites results
in reduced fitness of animals because of ionic imbalance of extra cellular fluids or increased
energy costs in the process of maintaining additional transport sites (Harris and Coley, 1991).
Rouse *et al*. (1995) presented that 24-hour exposure to nitrite concentrations of 0.4 mg·L⁻¹
and 0.6 mg·L⁻¹ decreased subsequent growth of redclaw crayfish (*Cherax quadricarinatus*)
by 17% and 67% and increased mortality by 5% and 48%, respectively. It can be expected
that it is a result of the stress reaction caused by nitrite treatment.

Finally, we can safely claim that the presented method of stress monitoring can and should
be used to fill knowledge gaps about sublethal effect of toxicants in aquatic environment and
give real levels of chemicals which do not cause any disruptions or deteriorations in health,
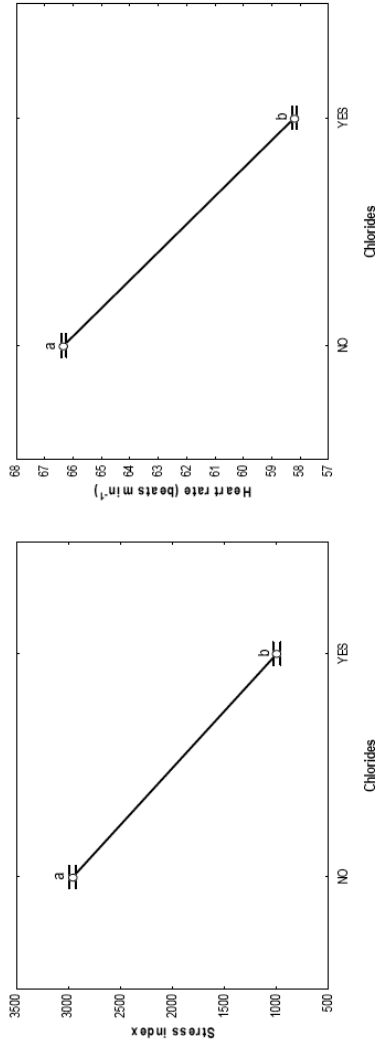behaviour or chemical communication of cultured or wild (free living) organisms.

Figure 7
The comparison of mean values (heart rate, stress index) reached in group with (400 mg Cl⁻·L⁻¹) and without an addition of chlorides. Data are presented as mean ± SE. Different superscripts differ at α = 0.05 (ANOVA, F = 2345.5, P < 10⁻⁶).

Figure 7
Comparaison des valeurs moyennes (rythme cardiaque, indice de stress) des écrevisses soumises à différentes concentrations de nitrite entre le groupe dans l'eau avec chlorures (400 mg Cl⁻·L⁻¹) et celui sans addition de chlorures. Les données sont des moyennes avec écart-type. Les différentes lettres diffèrent au niveau α = 0,05 (ANOVA, F = 2345,5 ; P < 10⁻⁶).
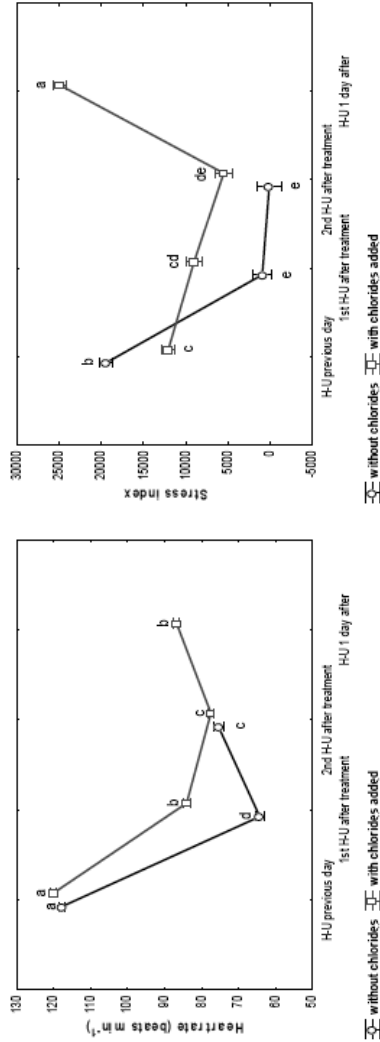
05p9

Figure 8
The comparison of values (heart rate, stress index) reached in group with (400 mg Cl⁻·L⁻¹) and without an addition of chlorides during particular hang-ups (H-U). Data are presented as mean ± SE. Different superscripts differ at α = 0.05 (ANOVA, F = 42.3, $P < 10^{-6}$).

Figure 8
Comparaison des valeurs (rythme cardiaque, indice de stress) atteintes par des écrevisses soumises à différentes concentrations de nitrite entre le groupe dans l'eau avec chlorures (400 mg Cl⁻·L⁻¹) et celui sans addition de chlorures pendant les moments de suspension de l'écrevisse. Les données sont des moyennes avec écart-type. Les différentes lettres diffèrent au niveau α = 0,05 (ANOVA, F = 42,3 ; $P < 10^{-6}$).

ACKNOWLEDGEMENTS

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RESEARCH ARTICLE

Investigation of chloramine-T impact on crayfish *Astacus leptodactylus* (Esch., 1823) cardiac activity

Iryna Kuklina · Svetlana Sladkova · Antonín Kouba · Sergey Kholodkevich · Pavel Kozák

Abstract The crayfish play an essential role in the biomonitoring and may reflect ambient water quality through the biochemical, behavioural and physiological reactions. To assess whether narrow-clawed crayfish *Astacus leptodactylus* can respond by heart rate changes to presence in water of such biocide as chloramine-T, adult males were exposed to its low (2 and 5 mg L^{-1}), moderate (10 mg L^{-1}, commonly used in industry and aquaculture) and exceeded (20 and 50 mg L^{-1}) concentrations. In addition, a physical stress test evaluated energy expenditure following the chemical trials. Three key reactions (cardiac initial, first-hour and daily prolonged exposure) were discussed with particular focus on crayfish initial reaction as the most meaningful in on-line water quality biomonitoring. After short-term exposure to both chloramine-T concentrations, crayfish were found to respond rapidly, within 2–5 min. According to heart rate changes, the 1-h exposure did not adversely affect crayfish at either concentration, as well as during daily exposure to 10 mg L^{-1}. As assessed by the heart rate, the 24-h exposure to 50 mg L^{-1} of chloramine-T was toxic for crayfish and led to substantial loss of energy that became apparent during subsequently conducted physical stress. The results supported a hypothesis that crayfish vital functions are connected with environment they inhabit closely enough to serve as biological monitors. Crayfish were tolerant to short-term chloramine-T exposure, while rapid crayfish reaction to an increased chemical level indicated their high sensitivity, an essential attribute of real-time environmental assessment.

Keywords Bioindicator · Biomonitoring · Heart rate · Narrow-clawed crayfish · Water treatment

Introduction

Freshwater crayfish, as representatives of aquatic invertebrates, are well studied biologically and ecologically. A significant area of research is the crayfish heart rate (Fedotov et al. 2000; Li et al. 2000) and their cardiovascular system in general (Wilkens 1995). Crayfish cardiac activity, which is directly related to circadian rhythm and locomotor activity, is affected, not only by environmental pollutants, but by external factors such as light (Udalova et al. 2009) and temperature fluctuations (Bojsen et al. 1998). Study of the cardiac reactions of crayfish and other aquatic organisms to environmental changes is of importance, since heart rate (HR) reflects the cumulative impact of stressors on organisms and may indicate whether their energy levels are sufficient to allow them to feed, grow and reproduce normally (Depledge and Galloway 2005). Thus, HR is an indicator of the crayfish state, while the crayfish state is an indicator of ambient water quality.

While most contaminants in natural waters can be identified through chemical analyses (Cleuvers 2003; Meinertz et al. 1999), it is valuable to know whether animals are able to detect contaminants in water. It has been suggested that crayfish, as well as fish, show avoidance reactions to harmful

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substances (Scott and Sloman 2004). This depends on the animal having sufficient space to escape (Gadzała-Kopciuch et al. 2004). In widely contaminated areas, in limited space, or in other conditions preventing avoidance of threat, avoidance behaviour is not manifested, and animals remain apparently stationary (Li et al. 2000). In such circumstances, variability in HR serves as one of the most reliable indicators of alarm and, hence, potential water contamination.

Through cardiac responses, crayfish can signal the presence of such pollutants as heavy metals (Styrishave et al. 1995), nitrites and nitrates (Jensen 1996) and ammonium compounds (Bloxham et al. 1999) as well as chemicals used for water treatment, such as chlorination and chloramination, and their byproducts. The compound used for chloramination, chloramine-T, is applied not only in industry (Moore and Calabrese 1980), but also in aquaculture for combating parasitic diseases (Harris et al. 2005). It is reported that chloramine-T, *N*-sodium-*N*-chloro-*p*-toluene sulfonamide (Meinertz et al. 1999), decomposes in water to paratoluene sulfonamide and sodium hypochlorite, the latter dissociating, to release free chlorine, ClO^-, which acts as an irritant to the fish, as well as killing parasites.

Extensive data describing the application of chloramine-T for prevention and treatment of parasitic diseases in fish are available (Altinok 2004; Athanassopoulou et al. 2009). Lake sturgeon *Acipenser fulvescens*, northern pike *Esox lucius*, walleye *Sander vitreus*, largemouth bass *Micropterus salmoides* and channel catfish *Ictalurus punctatus* have been exposed to varying concentrations of chloramine-T at different temperatures and durations. Chloramine-T treatment once a day for 1 h on four consecutive days at concentrations of 10–20 mg L^{-1} was not found to adversely affect survival of freshwater fish, in either cold or warm water (Gaikowski et al. 2008). At the same time, 10–50 mg L^{-1} of chloramine-T for treatment of amoebic gill disease in Atlantic salmon *Salmo salar* in seawater reduced parasite numbers (Powell and Clark 2004). Application of 50 mg L^{-1} of chloramine-T was toxic to eels *Anguilla anguilla*, although the concentration removed all trichodinids *Trichodina jadranica* from fish skin and gills (Madsen et al. 2000).

A study of rainbow trout *Oncorhynchus mykiss* exposed to 9 mg L^{-1} of chloramine-T for 45 min evaluated dorsal aortic pressure, ventilation frequency, HR, cardiac output and rate of oxygen uptake. Heart and ventilatory rates of fish were not affected, but oxygen uptake increased as a result of increased cardiac output (Powell and Perry 1999). Similar experiments with crustaceans are scarce. Speare et al. (1996) subjected American lobsters *Homarus americanus* to a 1-h exposure to 10 mg L^{-1} of chloramine-T. The experiment was conducted to assess the ability of chloramine-T to prevent ciliate parasites and to evaluate hemolymph biochemical and behavioural changes of animals with chloramine-T exposure. The disinfection procedure significantly decreased the number of parasites while not adversely affecting the lobsters.

For the present study, we selected crayfish based on the following reasons: crayfish are of increased interest in aquatic toxicological studies, while water treatment issue is of permanent interest too; no investigation was previously done to examine chloramine-T effect on the crayfish, water quality potential bioindicator. The primary aim of the current study was to compare the cardiac reaction of crayfish exposed to chloramine-T at acceptable levels and to the higher concentrations, associated with industrial use and aquaculture.

Materials and methods

The experiment was conducted in the Laboratory of Bioelectronic Methods for Geoecological Monitoring, Saint Petersburg Research Center for Ecological Safety of RAS, Russia, in July 2011.

Animals

The experimental group comprised 18 adult narrow-clawed crayfish *Astacus leptodactylus* males commercially obtained from Mostovoye Lake, Altai District, Russian Federation. Parameters included: total length, 109.3±7.2 mm (mean± s.d.); carapace length, 57.6±4.3 mm; weight, 33.6±2.0 g; HR at rest, 38±7 beats min^{-1} and regular circadian rhythms with 8–10-h nocturnal activity.

Equipment

A fibre-optic system for monitoring cardiac activity of benthic invertebrates (Fedotov et al. 2000) was used. As described by Kholodkevich et al. (2008), two thin optic fibre cables extending from a laser fibre-optic photo-plethysmograph connect a small optical sensor containing an infrared-light laser to the crayfish carapace. The heart is illuminated by an infrared laser beam formed in the photo-plethysmograph and transmitted by the first cable, and the signal containing information about heart activity is transmitted to the photo-plethysmograph by the second cable. After appropriate amplification, filtration and transformation to digital form, the cardiac signal is converted in the photo-plethysmogram and sent to a computer for analysis of crayfish heart activity that can be described by recorded HR (Kholodkevich et al. 2008).

Experimental design

A week prior to exposure, small connecters were attached to the dorsal side of the crayfish carapace directly over the heart (Fig. 1), using two-component fast fastening epoxy adhesive Poxipol, for connection to the measuring equipment. Crayfish were kept in a shared non-transparent aquarium with shelters and were fed twice a week on *Chironomidae* larvae
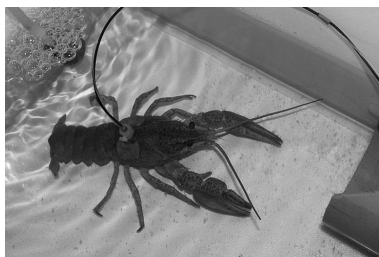
Fig. 1 Narrow-clawed crayfish *Astacus leptodactylus* with attached sensor for the heart rate registration

(approximately 1.5−1.7 g of chironomids per crayfish, food amount was based as 5 % of the crayfish weight). Three days before first exposure, six animals were moved for acclimatization to the experimental conditions of individual 20-L (water volume 6 L) glass non-transparent aquaria with a shelter in one corner and a water circulation pump in another. Throughout the experimental period, water temperature was 16–18 °C, pH was 7.2−7.4, dissolved oxygen level was higher than 6 mg L^{-1}, total hardness was 40–42 mg L^{-1} as $CaCO_3$ and nitrates content was less than 40 mg L^{-1}.

Five concentrations of chloramine-T were tested:

One group of three crayfish was exposed to 2 mg L^{-1} of chloramine-T for 1 h;
One group of three crayfish was exposed to 5 mg L^{-1} for 1 h;
One group of three crayfish was exposed to 20 mg L^{-1} for 1 h;
One group of three crayfish was exposed to 10 mg L^{-1} for 1 h and repeatedly, in 2 days, exposed to the same concentration for 1 h; one group of three crayfish was exposed to 10 mg L^{-1} for 24 h;
One group of three crayfish was exposed to 50 mg L^{-1} for 1 h and in 2 days exposed to the same concentration for 1 h; one group of three crayfish was exposed to 50 mg L^{-1} for 24 h.

Chemical trials were conducted every other day, and after each trial, exposed animals were returned to a shared aquarium and replaced by unexposed crayfish for the next treatments. Crayfish that were used for 24-h exposure were maintained separately and were not exposed to either chloramine-T concentration before treatment day. For each experimental trial, three control crayfish were selected from those kept in a shared aquarium and maintained in de-chlorinated tap water for 2 days, until exposure. During each trial, control crayfish were treated with clean water and were monitored to control that HR changes were not caused by manipulations. All animals were marked with individual numbers by permanent

water-resistant marker to avoid mixing and were not fed during the exposure period.

Prior to exposure, a solution of chloramine-T was tested for stability of active ClO^-, since it is suggested that this compound acts with better disinfection powers (Deinzer et al. 1978); thus, ClO^- acts as the main irritant to organisms. A control was conducted to ensure that concentration of ClO^- remained consistent during the exposure period. Stability of ClO^- in chloraminated water was tested by standard titration of potassium iodide with sodium thiosulfate using potato starch as indicator (Willson 1935). The test was repeated each hour for 12 h and repeated after a subsequent 12 h. Within 24 h, the concentration of chlorine in an aquarium containing crayfish that was not included either in the trial did not vary.

Before addition to test aquaria, chloramine-T was pre-dissolved in 0.3 L of distilled water. Chloraminated water (0.1 L) and de-chlorinated tap water (0.1 L) were added with flow rate of 3 L h^{-1} to each aquarium containing an exposure crayfish and to each control aquarium via plastic tanks suspended above aquaria and connected with pumps to add chloramine-T solution and water without disturbing the crayfish.

To assess crayfish energy expenditure, a stress test was conducted. Two hours after exposure, the crayfish were suspended and fixed by a fibre optic cable above the bottom of the aquarium for 1 h, so that water covered the carapace, but animals could not touch the bottom with their walking legs. Crayfish energy expenditure was assessed according to the HR change during their suspended state.

No mortality occurred during the experiment or in the days following exposure.

Heart rate measurement

Crayfish HR was continuously recorded for the whole experimental period. To evaluate cardiac reaction, periods of 1 h before treatment initiation and 1 h during chloramine-T exposure were considered. HR was sampled six times per minute and then averaged to 1 measurement per minute for statistical analysis.

Statistical analysis

Heart rate data were evaluated using STATISTICA v.10 software for Windows (StatSoft, Czech Republic). To evaluate the primary effect of chloramine-T exposure on cardiac activity, the HR recorded within 1-h pre-exposure was compared with the HR registered within 1 h of exposure using a paired *t* test for dependent samples. To evaluate effects of tested concentrations compared to control groups, separate *t* tests for independent variables were applied. HR changes were considered to be significant when $P < 0.05$.

Table 1 Heart rate (mean±s.d.) of crayfish (Cr, $n=18$) exposed for 1 h to selected chloramine-T concentrations (Cl-T, mg L^{-1})

Cl-T	2	5	20	10^a	10^b	10^c	50^a	50^b	50^c
Cr1	50±5 49±5								
Cr2	40±5 42±6								
Cr3	51±6 57±7								
Cr4		47±4 56±6							
Cr5		40±3 43±5							
Cr6		41±5 41±4							
Cr7			40±2 45±7						
Cr8			45±2 60±7						
Cr9			44±6 45±6						
Cr10				45±2 52±10	45±2 50±3		56±4 56±6	53±4 55±11	
Cr11				43±2 51±11	56±3 59±6		52±4 57±5	54±2 61±6	
Cr12				42±1 67±13	43±2 44±2		50±7 90±29	44±3 54±7	
Cr13						49±5 70±8			
Cr14						51±5 61±8			
Cr15						48±3 66±4			
Cr16									56±2 102±19
Cr17									52±5 83±20
Cr18									52±4 82±12

Left columns correspond to crayfish heart rate in pre-exposed state and right columns correspond to the heart rate over exposure period

[a] One-hour single exposure

[b] One-hour repeated exposure

[c] Heart rate over 1 h from 24-h exposure

Results

Chloramine-T exposure−chemical stress

Crayfish cardiac reactions to concentrations 2, 5 and 20 mg L^{-1} of chloramine-T appeared as follows: Crayfish exposed to 2 mg L^{-1} of chloramine-T did not show a significant increase in mean HR (48 beats min^{-1} before and over treatment period), and only one out of three crayfish showed slight cardiac reaction (Table 1). Very similar picture was observed when crayfish were exposed to 5 mg L^{-1} of chloramine-T. Mean HR did not significantly increase over the treatment hour (43 beats min^{-1} before versus 47 beats min^{-1} during chloramine-T exposure), while two out of three crayfish showed marked cardiac reaction to this concentration (Table 1). Crayfish HR was significantly disturbed when they were exposed to 20 mg L^{-1} of chloramine-T. All crayfish from this group reacted to presence of the chemical (Table 1), and mean HR increased from 43 beats min^{-1} at rest to 50 beats min^{-1} during chloramine-T exposure.

HR of crayfish under impact of 10 and 50 mg L^{-1} of chloramine-T was as follows: there were demonstrable differences in the heartbeat depending on concentrations (10 mg L^{-1} compared with 50 mg L^{-1}) and exposure duration (1 h compared with 24 h). HR of crayfish exposed to 10 mg L^{-1} of chloramine-T showed an increase within 2 min

of exposure and varied during the treatment hour with harmonic behaviour, fluctuating around a mean HR value of 57± 11 beats min^{-1} (Fig. 2). HR of the control group remained undisturbed, confirming that crayfish reacted to the chemical impact only and were unaffected by manipulation (Fig. 2).

The HR did not reach the significant increase (48 beats min^{-1} at rest versus 51 beats min^{-1} over exposure time) when crayfish were repeatedly exposed to 10 mg L^{-1} of chloramine-T (Fig. 3a). Conversely, over the first hour of the prolonged exposure to 10 mg L^{-1}, the crayfish cardiac reaction was significant compared to the pre-exposure state (49 and 66 beats min^{-1}, respectively), and the reaction was significant compared to the previous exposure to the same concentration (57 and 66 beats min^{-1}, respectively) (Table 1).

At the higher concentration (50 mg L^{-1}) of chloramine-T, a stepwise cardiac reaction with increase in HR was monitored within 5 min of initiating exposure. After the initial increase, a gradual decline in HR to the baseline level was observed; after which, a second significant HR increase was observed, continuing until the conclusion of the trial (Fig. 4). Mean HR over the treatment hour was 68 beats min^{-1} (compared to 53 beats min^{-1} at rest), while mean HR during repeated 1-h exposure to 50 mg L^{-1} significantly differed from pre-exposure state (50 beats min^{-1}) and reached level of 57 beats min^{-1} (Fig. 3b). With greater difference, crayfish HR significantly increased over long-term exposure to 50 mg L^{-1} of chloramine-T (first
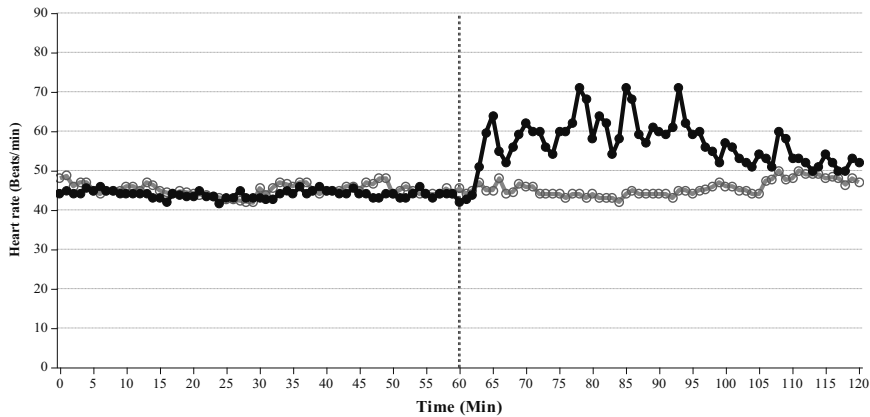
Fig. 2 Mean heart rate of crayfish exposed to 10 mg L^{-1} of chloramine-T ($n=3$, *black line*) and crayfish from control group ($n=3$, *grey line*). The heart rate is shown within 1 h before exposure initiation and over 1 h of treatment. In the figure, 60th minute corresponds to the moment of exposure initiation

treatment hour was evaluated), where HR reached 89 beats min^{-1} compared with 53 beats min^{-1} at rest state (Table 1).

For comparative demonstration, mean HR of each treatment group over pre-exposure and over exposure periods are presented in Fig. 5.

Suspension−physical stress

The stress test with suspension by optic cable showed both crayfish exposed to 10 mg L^{-1} of chloramine-T (for 1 h and for 24 h) and unexposed crayfish to maintain the HR at high level during the suspended state (107±8, 103±7 and 121±13

beats min^{-1}, respectively) (Table 2). In contrast, crayfish suspended after 1 and 24 h of 50 mg L^{-1} chloramine-T exposure exhibited a gradually decreasing HR during the stress test (101±9 and 92±7 beat min^{-1}, respectively) (Table 2, Fig. 6).

Discussion

The crayfish cardiac reaction during second exposure to both 10 and 50 mg L^{-1} of chloramine-T was expectedly less pronounced than during primary exposure to the same treatment dose. Data from aquaculture experience indicates that short-

Fig. 3 Crayfish heart rate ($n=3$, per each group) before and during primary (first exposure) and repeated (second exposure) 1-h chloramine-T treatment:
a 10 mg L^{-1}, **b** 50 mg L^{-1}. *Grey columns* correspond to 1 h of pre-treatment state and *stripped columns* correspond to the heart rate over 1 h of chemical treatment. *Column top* denotes heart rate mean value. *Whiskers* denote standard deviation
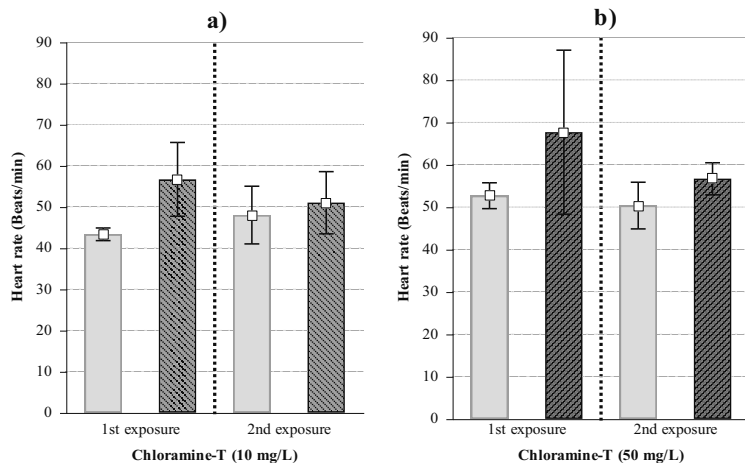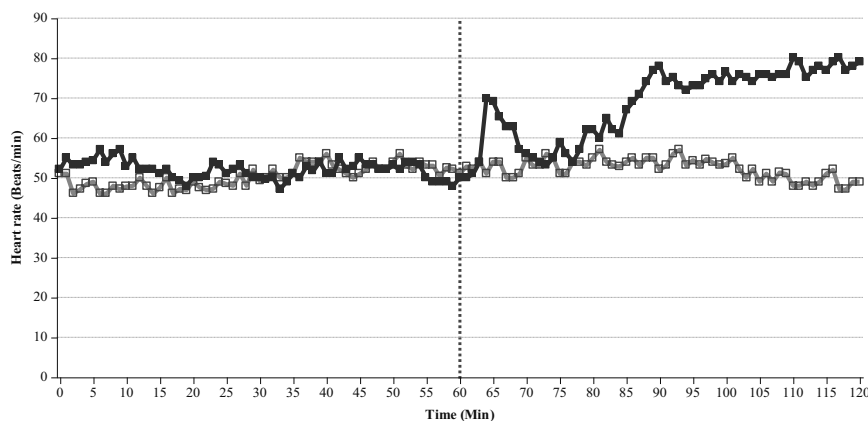
Fig. 4 Mean heart rate of crayfish exposed to 50 mg L^{-1} of chloramine-T (*n*=3, *black line*) and crayfish from control group (*n*=3, *grey line*). Figure illustrates the heart rate within 1 h before treatment initiation and over 1 h of treatment. 60th minute is the moment of exposure initiation

term treatment with chloramine-T at concentrations higher than 20 mg L^{-1} often appears harmful to fish (Gaikowski et al. 2008), while chloramine-T concentrations exceeding 40 mg L^{-1} are mentioned as those leading to mortalities in some cases (Madsen et al. 2000). In our trials, neither concentration was associated with apparent adverse effects to crayfish reflected in HR changes. The latter can be explained by the experimental design, where crayfish were exposed to a progressive increase in chloramine-T concentration. Such trials may have led to a damaging effect on crayfish chemoreceptors, which primarily sense chemical stimuli. Thus, the increase in HR at the same concentrations was observed different, with almost no increase during repeated exposure, even after few days of rest in plain water.

The so-called second wave of HR alterations that was observed with exposure to the higher concentration (Fig. 4)
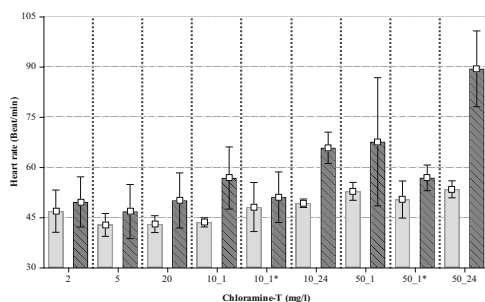

Fig. 5 Mean heart rate of crayfish (*n*=3, per each group) in pre-exposed (*grey columns*) state and over exposure period (*stripped columns*). *Column top* corresponds to mean value and *whiskers* denote standard deviation. Ordinate axis begins from level of 30 beats min^{-1}. *[1]* 1-h single exposure, *[1]** 1-h repeated exposure, *[24]* heart rate over 1 h from 24-h exposure

has been previously reported. Kuznetsova et al. (2010) monitored a second level of HR reaction following exposure of crayfish to hydroquinone (1,000 mg L^{-1}), where the second wave was indicative of a toxic effect. Kozák et al. (2009) reported a second wave of HR with exposure of crayfish to high concentrations of NaCl (>1,600 mg L^{-1}), when the HR increased slightly immediately following addition of the active substance, with a subsequent significant increase after a longer period of exposure. It should be noted that both chloramine-T and NaCl are commonly used parasiticides, and application of NaCl has been also reported as favourable for crayfish exposed to harmful nitrite concentrations of 60 mg L^{-1} (Kozák et al. 2005, 2011). The second wave of increased HR level confirms a toxic effect in the crayfish and indicates conditions under which crayfish could not maintain balanced homeostasis. Such a secondary increase in HR might be also caused by an increase of hypochlorous acid, which is commonly released together with ClO$^-$ by chloramine-T at pH 6–8 (Deinzer et al. 1978).

Visual observations allowed monitoring of movement of the optical fibres attached to the crayfish. The movement of

Table 2 Increase of crayfish (*n*=3 for each group) heart rate (mean±s.d.) under physical stress

	Untreated	10[a]	10[b]	50[a]	50[b]
Before physical stress	48±3	53±7	54±11	54±5	56±12
During physical stress	121±13	107±8	103±7	101±9	92±7
Increase percentage, %	150	102	90	90	65

[a] Crayfish were suspended after 1-h chloramine-T exposure followed by 2-h rest in clean water

[b] Crayfish were suspended after 24-h chloramine-T exposure followed by 2-h rest in clean water

Author's personal copy

fibres, confirming movement of the crayfish, may mean chemical disturbance only, but the question of whether HR increased as a result of chloramine-T exposure or initiation of movement, or both, remains open; however, it is doubtless that exactly chloramine-T affects both the crayfish state and behaviour, since mentioned reactions were not manifested by control crayfish (Figs. 2 and 4).

The crayfish exposed to certain chemical agents are affected, not in terms of cardiac activity alone, but respiratory and digestive function as well. Together with the cardiac system, these systems play essential roles for the living organism and present the full picture of animal functional state, although we cannot assess them directly in real time, as we can assess the HR. Depledge and Galloway (2005) suggested that cardiac activity within the normal range gives evidence of the proper physiological state of animals. In other words, HR in normal range indicates that organisms possess sufficient energy to maintain their internal processes. Therefore, we were interested in whether the crayfish lose energy subsequent to chloramine-T exposure and established the stress test with suspension by optical fibre. From this, we concluded that 1 h of chemical exposure did not intensively affect the crayfish state, since crayfish had energy enough to withstand the suspended state, i.e. they maintained a high HR. This does not mean however that low concentrations and short exposure periods did not affect crayfish, but harm was not evident in this case. After exposure to high, 50 mg L^{-1}, chloramine-T concentration, even after 1 h, the negative effect was obvious, since exhaustion of energy was indicated by the inability of crayfish to increase HR (Fig. 6). Similar results were reported by Bamber and Depledge (1997) while

evaluating the functional state of shore crabs *Carcinus maenas* taken from several contaminated localities. When the crabs were taken from rivers with poor water quality, they also showed declining HR when exposed to physical stress.

Conclusion

This study demonstrates that narrow-clawed crayfish reacted within several minutes of initiation of exposure to chloramine-T dissolved in water. Such rapid reaction underlines high crayfish sensitivity, an important factor in biosensors selecting for real-time water quality monitoring. At the same time, crayfish showed good tolerance to short-term chloramine-T action, increased their HR at a relatively low level when were chemically exposed and kept high HR during the physical stress. This study primarily addressed crayfish alarm reaction that means the average increase of the HR in the first hour of exposure. We did not consider peculiarities of physiological reactions, since they cannot be used for the on-line monitoring that is potential application of this study. Although we are unable to concretely define single chemicals based on cardiac alterations only, it is important to know possible reactions of crayfish to certain chemicals often applied to aquatic environment and those potentially unsafe for aquatic life. The results might serve a relevant basis for further studies aimed to establish other effects of chloramine-T exposure to crayfish as a representative of freshwater environment and important bioindicator for its quality monitoring.
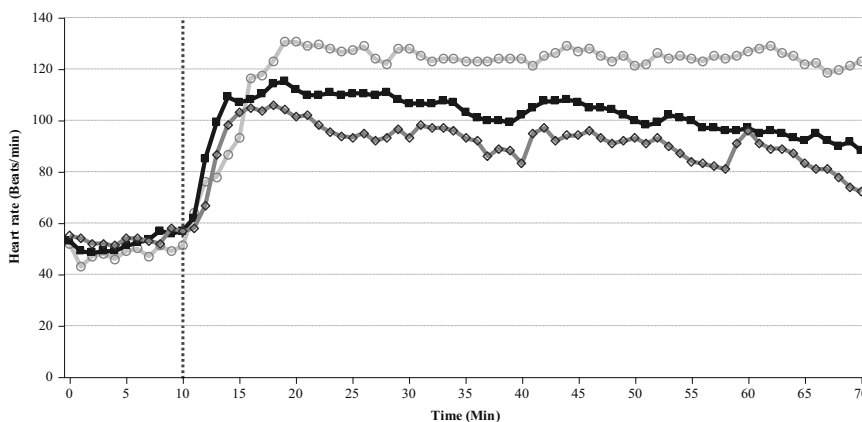

Fig. 6 Mean heart rate of crayfish ($n=3$, per each group) under physical stress. *Grey "bubbled" line* denotes heart rate of unexposed crayfish, when their rest state is followed by 1-h physical stress (suspension). *Black line* corresponds to heart rate of crayfish suspended for 1 h after 1-h exposure to 50 mg L^{-1} of chloramine-T followed by 2-h rest. *Grey "rhombic" line* corresponds to heart rate of crayfish exposed for 24 h to 50 mg L^{-1} of chloramine-T and after 2 h of rest exposed to physical stress for 1 h. In each event, the suspension starts from 10th minute

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CHAPTER 3

DEVELOPMENT OF THE SYSTEM FOR BIOLOGICAL MONITORING

3.1. Registration of cardiac activity using noninvasive technique

3.2. Detection of movement using video monitoring

3.1. REGISTRATION OF CARDIAC ACTIVITY USING NONINVASIVE TECHNIQUE

Description of biomonitoring system

The applications of patents entitled "Method of ethological monitoring of crustaceans and/or molluscs and ethological system for monitoring of crustaceans and/or molluscs behaviour" and "Sensor for measurement of crayfish heart rate" have been submitted 29.04.2014. "Noninvasive sensor" from 28.05.2014 has been registered in Patents and Utility Models Database Report.

Present monitoring system is based on noninvasive sensing element (*sensor*) (Figure 1) which transmits information about cardiac activity of crayfish and other benthic macroinvertebrates possessing sufficiently large exoskeleton for sensors attachment and carrying – decapod crustaceans and bivalve molluscs. The sensor is a component part of the whole *biomonitoring system for analysis of crayfish cardiac activity for evaluation of water quality*. The sensor itself is an electronic system which enables infrared light irradiation and measurement of the part of reflected light from the study object. The principle of cardiac activity measurement is based on reflection of infrared light from the crayfish hemolymph containing in the heart muscle and changing during each heart contraction. The sensor is noninvasively attached to the crayfish carapace dorsal side (using two-component fast fastening epoxy adhesive) over the place of heart location (Figure 2). The sensor illuminates infrared light towards to heart area and measures the reflected light. Intensity of the backward light depends on hemolymph volume in the heart and can be converted to electric signal. Strain of this signal is directly proportional to intensity of reflected light and amount of hemolymph in crayfish heart. In such indirect way, the crayfish cardiac activity (heart rate) is measured.
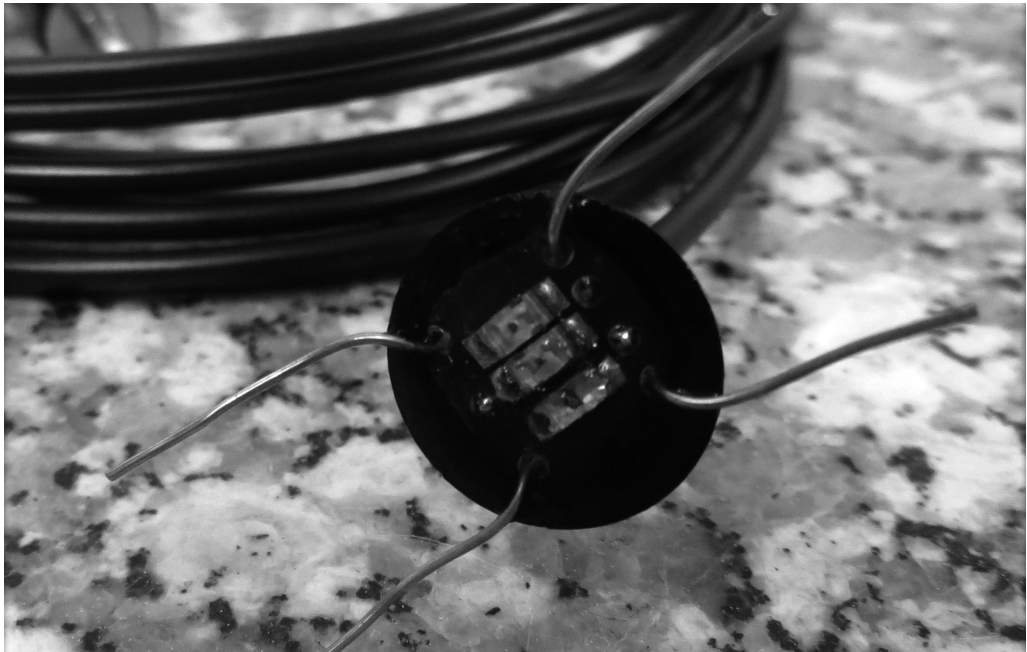

Figure 1. *Noninvasive sensor for registration of cardiac activity of benthic crustaceans possessing hard external carapace.*

Among biggest benefits of such sensor are:
- fast noninvasive, (within minutes but depends on outside temperature and humidity), fastening and simple fast, within seconds, detachment to the crayfish (potentially other hard-shell decapod crustaceans and bivalves with sufficiently large body size: crabs, shrimps, mussels);
- fast, within few days, adaptation to carry sensor on the carapace;
- continuous real-time recording of the cardiac rate (eventually ventilatory rate);
- unrestrained animals movement (depends just on aquarium size);
- unaffected vital functions, such as growth (reflected in successful moulting), feeding, nocturnal rhythmicity and general behavioural manifestations.

Figure 2. *Signal crayfish (Pacifastacus leniusculus) with attached sensor for cardiac activity registration.*

The whole biomonitoring system (Figure 3) is comprised of 12 aquariums and is able to monitor 12 crayfish simultaneously. Each aquarium is equipped with a shelter. To avoid additional disturbances of crayfish state and video detection process, water aeration is set inside upper water storage tanks. Set of 3 aquariums is replicated 4 times. Set of 6 aquariums is run by 1 camera. The whole system is equipped by peristaltic pump, which enables to deliver treatments insight aquariums at very low pumping rates without crayfish disturbance. The system is equipped with automatic illumination system (from lightening to darkening), with possibility to keep any necessary light regime.

Figure 3. *Experimental biomonitoring system for noninvasive recording and analysis of crayfish cardiac and behavioural activities.*

3.2. DETECTION OF MOVEMENT USING VIDEO MONITORING

Description

The system for detection of the crayfish locomotor activity utilises standard internet protocol (IP) video camera. Camera is fixed in such position and objective angle is selected so, that the camera can operate 6 aquariums (i.e. 6 crayfish) simultaneously. Also, IP camera enables distant (through the internet) monitoring of crayfish movement, what is of big use when there is no possibility to control long-time investigation during whole experimental period. System enables to the user to mark certain area of each single bottom of aquariums and then connect it with recording of cardiac activity using the software. System processes video recording in real time and detects the movement of single crayfish. Image of each aquarium is analysed independently. Approach of background dynamic assessment is used for detection of movement. Due to evaluated background, it is then possible to mark out in each image its changes and thus detect present crayfish position. The sensitivity of movement detection can vary from detection of movement of single leg up to total change of crayfish position. Simultaneously, video recording system provides information on single specimens movement to the system of cardiac activity analysis. The view of crayfish from below of aquariums during monitoring is shown in Figure 1.

Figure 1. *Detection of the crayfish movement during biomonitoring. Crayfish body parts moving at the present moment are automatically marked by the red colour.*

Conclusions

The presented biomonitoring system is easy to manipulate and to process the experimental data. What is also important to note that the system demands low costs for its set up and operation. The main advantage of present biomonitoring system is the simultaneous combination of units for recording and analysis of cardiac activity at once with the unit for detection of movement. Due to such combination, it is possible to eliminate impact of crayfish movement on its cardiac activity when analysing the heart rate changes caused by environmental disruptions. In order to draw broader list of potential bioindicators and thus form more relevant data on the water quality and aquatic biota state, one should incorporate other hard-shell macro invertebrates (crabs, shrimps, mussels) into discussed biomonitoring approach.

Potential application

When the system is fully developed and is completed by threshold to signal an alarm conditions, it can be potentially used at such municipal industrial facilities as water treatment (effluent) and water supply (both influent and effluent) stations for water quality continuous monitoring (Figure 2).
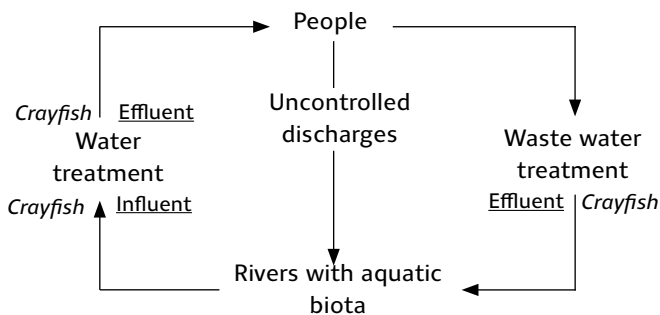

Figure 2. *Crayfish possible placements at water treatment and supply facilities.*

CHAPTER 4

FIRST EXPERIMENTAL TRIALS WITH BIOMONITORING SYSTEM

4.1.   Crayfish cardiac and behavioural responses to chemical and natural stimuli

4.1. CRAYFISH CARDIAC AND BEHAVIOURAL RESPONSES TO CHEMICAL AND NATURAL STIMULI

Introduction

Aquatic animals can detect, discriminate, and sensitively respond to a variety of both beneficial and harmful chemicals, as well as other natural odours and cues which present in water (Derby and Sorensen, 2008). Therefore using freshwater animals as bioindicators of environments they inhabit is among the top-approaches in eco-biological studies nowadays. Behavioural and physiological responses are the function of crayfish environmental adaptation (Burmistrov and Shuranova, 1996). Such crayfish responses can (and should) be considered under exposure to various stressors, in order to know more about crayfish ethophysiological capacity and limits in relation to ambient stress. In the broader sense, when ambient conditions are negatively changing, the crayfish are tending to adapt, demonstrating alterations in locomotor, cardiac and ventilatory activities which can be symbolically divided into physiological (Burmistrov and Shuranova, 2010; Schapker et al., 2002) and ethological (Shuranova and Burmistrov, 2010) responses.

Vital behavioural alterations (feeding, locomotion and defence) associated with predatory fish and conspecific crayfish odours have been successfully investigated by Gherardi et al., 2011 on an example of invasive red-swamp crayfish *Procambarus clarkii*. The results showed crayfish to be more responsive to conspecific alarm odour rather than to odour of potential predators. However visible behavioural changes often may be not manifested even when crayfish are significantly disturbed (Li et al., 2000; Schapker et al., 2002). Thus, observing ethological reactions might be not enough when evaluating crayfish ambient conditions, while measuring certain physiological factor at once with behaviour might help to illustrate real crayfish state under some exposure. In this pilot study, we examined recently developed biomonitoring system using another invasive crayfish species – signal crayfish, *Pacifastacus leniusculus*. We tested range of odours which are believed to play the most meaningful roles in crayfish environment, plus one chemical odour, that was tested to examine signal crayfish suitability for water quality biomonitoring.

Material and methods

Experimental trials

Using noninvasive method for heart rate registration in combination with movement video tracking, the impact of the following odours on signal crayfish were investigated:
   1) odour of food (milled *Chironomidae* larvae) after one day of starvation;
   2) odour of active predator (European perch, *Perca fluviatilis*);
   3) odour of conspecific males and females;
   4) odour of conspecific crayfish injured by thoracic leg ablation;
   5) odour of chloramine-T (industrial common disinfectant and parasiticide).

Experimental animals

The crayfish were caught in baited traps in early June 2013 in Vysočina Region, Czech Republic. The experimental group, comprised 12 adult individuals of both sexes (6 males, 6 females), was formed based on the following parameters: weight, 56 ± 6.1 g; carapace length, 54 ± 2 mm; and total length, 108 ± 4 mm (mean ± standard deviation).

Experimental design

The experiment was conducted in August 2013. For acclimatization, crayfish were maintained during one week in individual 10 L non-transparent glass aquaria, each equipped with a shelter. Prior to manipulations, selected crayfish were marked by permanent water-resistant marker with individual numbers to avoid mixing or repeated animals exposure. A week prior to recordings, the noninvasive sensors were fastened to the dorsal side of the crayfish carapace directly over the heart using two-component fast fastening epoxy adhesive. Throughout the experimental period, water temperature was 19–21 °C, pH was 7.2–7.4, dissolved oxygen level was higher than 6.5 mg L^{-1}. To avoid disruption of crayfish nocturnal rhythmicity, the illumination regime was set and automatically maintained at 12 h of light and 12 h of dark. Crayfish were fed twice a week on Chironomidae larvae. The remaining food and crayfish vital activity by-products were removed during the water exchange the next day after feeding. During the exposure trials animals were not fed.

Treatments

Odour of food was simulated by introducing to crayfish the smell of milled *Chironomidae* larvae. For this, 1 g of defrosted chironomids was milled and mixed with 1 L of distilled water, filtered, and then just water slightly coloured in reddish tint and containing food odour was used.

To obtain *predator odour*, adult pikeperch was kept for 24 h in isolated tank with 100 L of still water, and just prior to experimental trial fish water was collected and used.

To prepare *odour of conspecific crayfish*, 3 adult males and 3 adult females were kept in 50 L shared aquariums with still water for 24 h as well, and then this water was collected just before the trial and used for treatment.

To simulate the *odour of injured conspecific*, the crayfish was placed into separated aquarium filled with 5 L of water, and after that last thoracic leg was ablated, and crayfish was left in aquarium for 1 h. After that water was collected and experimental trial started immediately.

Chemical odour was simulated by chloramine-T, which was pre-dissolved in distilled water 1 h prior to exposure, covered and left in laboratory glass until the trial. During treatment, the final chloramine-T concentration in each aquarium formed 10 mg L^{-1}.

For each trial, treatments in amount of 50 ml were pumped at once into aquaria via peristaltic pump set at a flow rate of 50 ml min^{-1}. After finishing of each recording, the water in aquariums was exchanged. No mortality occurred during the experimental period and within days following the chemical exposure.

The crayfish cardiac and locomotor activities were recorded within half an hour before experimental trials and within half an hour after test-odours were introduced inside aquariums. Such a relatively short time period was chosen to examine crayfish ability to react fast on selected stimuli. In other words, the main interest was focused on crayfish primary reactions to test-odours.

Results and discussion

Crayfish cardiac and locomotor responses to tested odours

Numerical changes in heart rate occurred under impact of examined odours are presented in Tab. 1. During each trial the initiation of movement of several crayfish was noticed, but the locomotion activity was not manifested at once with the heart rate increase. An exception

was chemical treatment, chloramine-T, when the crayfish started movement simultaneously with increasing of the heart rate. That highlights tight relation of behavioural and cardiac activity, as well as stronger stimulating effect of the chemical stimulus. However, there is no evidence for having higher heart rate under chemical exposure than "natural" odours. Therefore food and injured crayfish odours were more intensive compared with chloramine, while predator and conspecific odours were much less disturbing in comparison to the chemical one. This might be explained from several points of view. First is that concentration 10 mg L^{-1} of chloramine-T is not that high, and even recommended for treatment of water for drinking within 1 h (Haneke, 2002). Although for crayfish even this concentration was enough to "recognize" it in water and signal its presence by heartbeat and motion alterations. Second is that water taken for testing of conspecific odour was obtained from both males and females. But when these odours were offered to both sexes, there was not difference between reactions, so that the results were pooled together. It is evidently that August, when current experiment was done, is not the period for signal crayfish reproduction, therefore this odour was not so intensive and attractive for tested crayfish. On the contrary, such pronounced reaction to odour of injured conspecific is apparently caused by the same reason as reaction to food odour. When crayfish walking leg was ablated, some amount of hemolymph was released into water along with injured conspecific pheromones. The crayfish are known to be omnivorous or carnivorous, but often they can manifest cannibalism under certain stress conditions (Nyström, 2002). This is likely to explain such significant heart rate elevation under exposure to injured conspecific odour. Increase of the locomotor activity under impact of stronger (primarily chemical) stressor is likely the demonstration of avoidance reaction of crayfish whose movement is restricted by aquarium size.

Table 1. *Responses of crayfish to tested odours.*

Odour	HR before, bpm	HR during, bpm	Δ, %	HR variability	Movement
Conspecifics	38.2	43.5	13.9	Stable	n/concurrent
Predator	39.2	48.5	23.7	Stable	n/concurrent
Chloramine-T	40.5	56.2	38.8	Increased	concurrent
Food	33.1	46.7	41.1	Increased	n/concurrent
Injured one	36.3	54.6	50.4	Increased	n/concurrent

* HR before – heart rate, calculated over 30 min before experimental trial; HR during – heart rate, calculated within 30 min of experimental trial; bpm – beats per minute, beat min^{-1}; Δ – percentage of the heart rate increase; HR variability – changes of standard deviation; Movement – concurrence of the moment when the heart rate increased with movement initiation; n/concurrent – non-concurrent.

An interesting observation should be noted, where crayfish were found to slow the heart rate during the first minute of reaction manifestation. This peculiarity must be taken into account and the heart rate measured within this short period should be likely excluded. Otherwise there is risk to average those values with the following heart rate and will not reveal any difference when evaluating results of experiment. Perhaps this is the only disadvantage of biomonitoring system at the moment: there is no set threshold of the heart rate exceeding which the system would signal about alarm. However this is the point for further investigations.

Effect of stimuli on crayfish behavioural patterns

   When exposed to different test-odours, male and female crayfish were observed to behave with different pathways, demonstrating differing scenarios of their reaction. Thus, females more preferred staying inside of shelters even when their heart rates increased, while male crayfish tended to move out of shelters after the moment when their heart rates increased as the response to introduced odour. If female crayfish with increased heart rate stayed inside of shelter, her cardiac activity characterised by wave-like heartbeat (Figure 1). However, if female moved out the shelter, the heart rate was stably high, and none of such animals returned back to the shelter. Identical with females, those males, which stayed inside of shelters after increase of the heart rate, had the wave-like heartbeat. However part of males moved out of their shelters, and they either demonstrated stable high heart rate (Figure 1) and stay outside or returned back to the shelters with wave-like heart rate as in the case when crayfish stayed in the shelter. Both male and female crayfish exibited higher locomotor and cardiac activities if they were already outside in the moment of treatment initiation. In spite of obvious disturbance, those crayfish did not tend to move inside of the shelters during the whole treatment period.
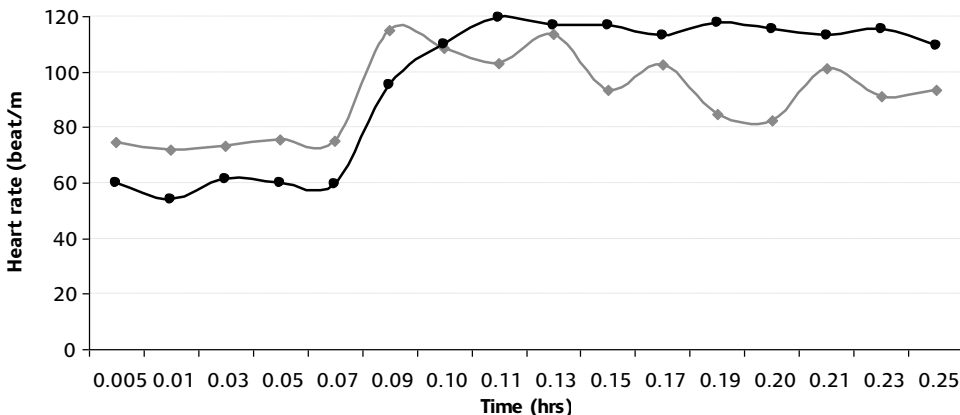

Figure 1. *Visualization of crayfish heartbeat pathways. Black line demonstrates stable high heart rate. Grey line demonstrates wave-like heart rate. Both curves describes crayfish reaction to the food odour.*

Peculiarities of the cardiac activity recording

   When analysing the "raw" heartbeat curves of crayfish at rest and in the moment of stimulus introduction, even visually there was possible to reveal the noticeable features. First is that heartbeat curve sometimes seems to have two beating frequencies. In Figure 2, the fragmentary cardiac activity of crayfish starting from the rest state (Figure 2a), going through excitation (Figure 2b,c) period and returning to quiet state (Figure 2d,e) is described. For peculiarity demonstration, the disturbance was caused by dropping the dropping one *Chironomidae* larva into aquarium with crayfish. In Figure 2a, there might be seen clear two peaks of the "heartbeat": one is more intensive ("sharper" and "higher") and second one is less intensive ("smaller"). When crayfish recognises the dropped larva, the abrupt changes occurs, and the heartbeat shape is changed and the heart rate is accelerated (Figure 2b). In Figure 2c, there is heartbeat of excited crayfish, eating the caught larva and having double elevated heart rate, in comparison to rest state. When excitation decreases, the heart rate

decreases as well, and the second peak of the heartbeat curve appears again (Figure 2d). In the last figure (Figure 2e), the heart rate returned to the norm and two peaks are clearly visible again.

It would be logically to suppose that we record something else at once with cardiac activity. Listerman et al. (2000) faced similar challenge when invasively recorded electrocardiograms in crayfish, and called this "phenomenon" electromyogram recording. Regardless the way of its obtaining, the myogram can have its place elsewhere as well. Just it is unclear which crayfish muscle exactly contributes to the cardiac activity. Muscles that parallel the heart on both sides were mentioned by Listerman et al. (2000). Vogt (2002) illustrates in detail the crayfish thoracic musculature, but does not mention the possible impact of one another. One might be possible to suppose that we observed impact of the muscle which is responsible for ventilatory activity, i.e. scaphognathite movement. This vital and extremely active process in crayfish is also very sensitive to environmental changes, even in tolerant species (Schapker et al., 2002). Under impact of certain stimuli ventilatory activity would have to increase as well, so that we would observe homogenous heartbeat curve. However when crayfish is in rest state, second peak in the heartbeat curve must appear much more frequent, since crayfish respiratory rate is known to be significantly higher than the cardiac rate (Vogt, 2002). Therefore if the second peak in the heartbeat curve would correspond to impact of respiratory activity, it must appear much frequent and pronounced.

Although McMahon and Wilkens (2012) discussed crustaceans in general, their explanation connected with epimeral muscles activity seems to be the most believable. These muscles were shown to contract following each heart beat of the lobster *Homarus americanus* (Wilkens and McMahon, 1972), and they likely play a significant role in ventilatory process of crustaceans with the similar structure, including freshwater crayfish (McMahon and Wilkens, 2012).
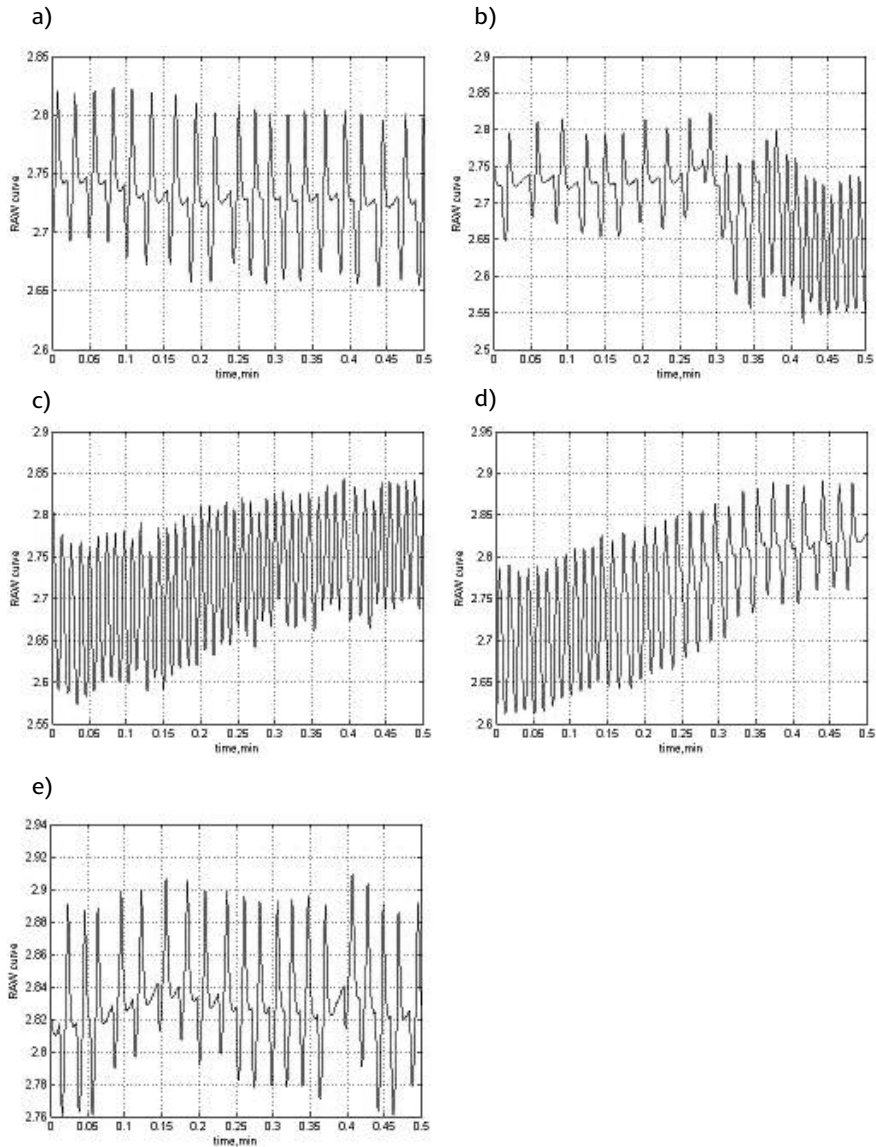
Figure 2. *Crayfish cardiac activity at different functional states, recorded along with impact of side-myogram – epimeral attractor muscle activity: a) rest state; b) initiation of disturbance; c) excited state; d) initiation of relaxation; e) quasi-relaxed state.*

Conclusions

Study of crayfish reactions under impact of different stressors is critical as it can help with the following:

- to examine crayfish primary reactions in relation to their environment;
- to learn more about crayfish ethophysiological state in different environmental and laboratory conditions;
- to distinguish crayfish responses to chemical stimuli from responses to natural cues;

- to investigate species-specific crayfish sensitivity in order to establish threshold levels of the heart rate before starting continuous monitoring;
- to implement more efficient control of the water condition and prevent in time its deterioration.

However there is remaining need to set up thresholds of crayfish cardiac reactions, which will enable to conclude with the confidence whether manifested reaction was evidence of "high disturbance" or was yet "acceptable".

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CHAPTER 5

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

It seems apparent that the most efficient biomonitoring study should involve more than one taxonomic group, in order to draw the comprehensive insight into the ecosystem's actual state. Along with crayfish, other macroinvertebrates, fish and algae are used as bioindicators for assessment of freshwater environment (Resh, 2008). Because using of mentioned aquatic communities can meet number of difficulties, crayfish seem to be more reliable biomonitoring tool. Macroinvertebrates quantitative sampling may be complicated because of their non-random distribution in the river bed, while some invertebrates life cycles seasonality impedes their occurrence at some times of the year (De Pauw et al., 2006). Some complications may occur when sampling algae, since often they are difficult to identify (Resh, 2008). Disadvantage of fish is that most of them are too motile and accumulate pollutants throughout significant areas (Resh, 2008). Therefore it is an issue, whether such results are relevant if taken alone. Moving across the water body in all dimensions, fish can "collect" either too high or too low concentration of certain substances, if swimming in narrowed area, therefore resulting level of accumulation can be irrelevant. In the contrary to fish, mussels are known to accumulate harmful substances up to very high level without depuration, so they may likely reflect quite irrelevant level of pollution. In contrast to other freshwater communities, crayfish are easy to identify (Pöckl et al., 2006) and catch (Policar and Kozák, 2005). Additionally, crayfish they are moving just in two dimensions most of the time, that provides nearly homogenous pollutant's accumulation over selected area of the bottom.

We should be careful yet when uncompromisingly asserting flexibility of crayfish as bioindicators, since it can be otherwise even if talking about native species. Addressing to Dorn and Wojdak (2004), crayfish may indicate rather declining of water conditions than prosperity, however most of authors reported that crayfish are often not that good bioindicators as it is commonly suggested (Demers et al., 2006; Füreder and Reynolds, 2003); nevertheless they are key-stone, pivotal freshwater species (Reynolds and Souty-Grosset, 2012).

According to the published literature, both native and non-native species were successfully used for behavioural and physiological investigations, whereas *P. clarkii* was the most popular laboratory object, in both georgraphical and methodological points of view (Tab. 1). What is surprising that *O. limosus*, causing one of the biggest threat to native European crayfish species, was used just once. However that might be good solution to use the most spread and "firm" species, which are abundant and tend to spread even even more in near future. On the other hand, wider use of *P. clarkii* could be explained by generally wider prevalence, bigger body size and longer life-span than in *O. limosus* (Kouba et al., 2013). Also, it is an issue whether to use so-called "sensitive" or "tolerant" species for the biomonitoring. On the one hand, more sensitive crayfish are of higher threat, but their susceptibility seems giving "better" results compared to less sensitive species. On the other hand, there is wider space for experimentation with well adapted alien crayfish due to wider availability and lower risk of immediate death in the beginning of some test. At the same time, indirect proof assumes: if certain tolerant species appears sensitive enough to indicate some stress stimulus, we can expect to see quite similar reaction from the side of sensitive species even under better conditions, because of lower "threshold" of sensitive crayfish. However each hypothesis demands an experimental proofs.

Apparently, it would be wrong to focus only one crayfish species in biomonitoring, since bioindicator selection, as well as biomonitoring approach, is a very ambiguous issue. On the one hand, we need sensitive, purely susceptible crayfish, which would not "miss" the moment of contamination when being "on guard" of the water quality. On the other hand, we need tolerant species, which would be able to withstand heavy contamination, and would not die

immediately, but on the contrary would be able for accumulation of harmful substances for further analysis. In view of the above, the most sensitive crayfish species (*A. astacus, A. leptodactylus, Austropotamobius* spp.) could be applied for biomonitoring in real time and for evaluation of "moderately-acceptable" environmental concentrations of potentially unsafe compounds. Whereas tolerant crayfish (*O. limosus, P. clarkii, P. leniusculus*) would be more appropriate for investigation of potentially lethal concentrations of toxic substances and for modelling of possible dangerous incidents in laboratory conditions. It is nessecary to examine both sides, in investigate if crayfish would be able to signal some critical occurrence in labs, and if it is worthwile to apply such approach in the field.

Such, depending on susceptibility to environmental conditions, nearly each crayfish species can be potentially involved into different kinds of biological monitoring, which is mainly aimed on the water quality control. However one should take into account specifications which can impact the results of investigation: day or night time, male or female specimen, repletion or hunger, growth or moulting, readiness for reproduction. These factors can affect locomotor, cardiac and ventilatory activities, which the ethophysiological biomonitoring is focused on.

It is shown that for the most efficient biomonitoring the combination of at least two parameters is essential. However, when combining physiological side with ethological, it is very likely to find out their interaction, which is very unwanted, although it can be avoided if handling carefully. Increased spontaneous locomotor activity affects the crayfish cardiac rate, therefore one must keep in mind: if crayfish movement initiated along with the heart rate increase under some impact, then the heart rate increase is likely irrelevant, since movement can contribute to the heart rate increase more than extrinsic stimulus. Because such effect is a very case-dependent, the precise analysis and detail comparison are always essential.

It was surprising to reveal that more intensive cardiac and locomotor activity is not the evidence of chemical impact rather than impact of some natural stimuli. However it is true that apparently toxic substance can be of lower effect than the common odour (Shuranova and Burmistrov, 2002). Therefore, it is important to test on crayfish broad range of the various odours and appropriate chemicals, in order to draw the list of hypothetical events where bioindicator-crayfish would be suitable.

Talking about cardiac reactions peculiarities, it is worth mentioning that each cardiac reaction is not primarily manifested in the heart rate increase, but the reverse effect is usually observed, because the heart rate tends to slightly decrease. In spite of quite short duration, just about one minute, the decrease phase may cause data divergency. Even if the heart rate increases within several minutes, we can simply lose this effect when averaging decrease and increase phases, or this difference can appear too unnoticeable. However, from the physiological point of view, such decelerating reaction is very important. The periods when crayfish stand motionless after introducing of some stress-factor can be symbolically called the orienting response. There might be drawn the parallel with the similar neural reaction of humans, when manifestation of such orientation response is needed to adapt to environmental changes (Burmistrov and Shuranova, 1996). Burmistrov and Shuranova (1996) also noted that the very rapid interruption of the motor activity might be demonstrated by crayfish prior to orienting response, however first and second reactions often may concur, especially under relatively slight disturbance. Therefore we did not reveal the first phase in our trials. Nevertheless, we always recognise third phase of the reaction, which is in fact the defence against environmental disruption, during which ethophysiological activity of crayfish is always excited.

Table 1. *Monitoring techniques ever investigated behavioural and physiological parameters of crayfish species examined with them.*

Species	Country	Technique	Reference
A. astacus	Denmark	Optical gate, PPG	Styrishave et al., 2007
	Russian Federation	PPG	Fedotov et al., 2006
A. leptodactylus	Russian Federation	PPG	Sladkova and Kholodkevich, 2011
	Russian Federation	ESG	Shuranova et al., 2003
C. destructor	USA	Camera-based tracking	Basil and Sandeman, 2000
	Australia	ECG	Goudkamp et al., 2004
		Camera-based tracking	Patullo et al., 2007
		Chelae force measurement	Seebacher and Wilson, 2006
E. sulcatus	Australia	Chelae force measurement, Radio telemetry	Lowe et al., 2010
O. australis packardi	USA	ECG, video recording	Bierbower et al., 2013
O. limosus	Czech Republic	Radio telemetry	Buřič et al., 2009
O. rusticus	USA	Camera-based tracking	Panksepp and Huber, 2004
O. virilis	USA	Camera-based tracking	Bergman and Moore, 2003
P. leniusculus	UK	PPG	Bloxham et al., 1999
	Denmark	Optical gate, PPG	Styrishave et al., 2007
P. clarkii	USA	ECG, ESG	Bierbower and Cooper, 2009
	Italy	PPG, SG PPG	Bini and Chelazzi, 2006
		Video tracking, Passive acoustic monitoring	Buscaino et al., 2012
	Russian Federation	PPG	Fedotov et al., 2006
	France	Camera-based tracking	Jamon and Clarac, 1995
P. cubensis	Russian Federation	Camera-based tracking	Burmistrov and Shuranova, 2010
		ESG, Optical gate	Shuranova and Burmistrov, 2010

* PPG – photoplethysmography; ECG – electrocardiogram; ESG – electroscaphognathitegram; SG PPG – scaphognathite-photoplethysmogram.

Conclusions and future perspectives

In the present thesis, the existing approaches for the water quality monitoring were reviewed and new rational developments were introduced. The proposed innovations are purposed to implement more efficient control for water conditions and prevent in time their sudden deterioration. Despite the various possibilities of analytical techniques, the most effective environmental monitoring is up to the aboriginal animals. Up to now, basically behavioural and biochemical characteristics have been used for evaluation of animals health state (including crayfish) and their ambient conditions quality. While using noninvasive approach for the heart rate measurement in combination with monitoring of crayfish behavioural activity opens new horizons in the biomonitoring expanse, since the developed biomonitoring system enables examination of particular characteristics of biology of crayfish, with additional possibility for incorporation other freshwater crustaceans and mussels as well.

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ENGLISH SUMMARY

Crayfish as bioindicators of water quality

Iryna Kuklina

In recent decades, the ecological status of the freshwater crayfish has changed drastically from a sensitive indicator of an aquatic environment to a tolerant species that can survive in a wide range of unfavourable conditions. Despite all controversies on being or not being proper bioindicators, crayfish are a key species that plays a crucial role in the freshwater ecosystem. Regardless of whether certain crayfish possess a particular environmental sensitivity or not, all species can be used in biomonitoring investigations. The main objectives of the present thesis were development and implementation of system for continuous monitoring of water quality using crayfish as the bioindicator. Being less complex than vertebrates (e.g., fish), but being sufficiently complex compared to some other hard-shell freshwater invertebrates (e.g., mussels), crayfish present a useful biomonitoring object, which is easy to manipulate with, and which provides experimental data which is easy to obtain, analyse and interpret.

The first part of this thesis is devoted to an evaluation of crayfish as suitable bioindicators. We showed that, when conducting the biomonitoring of metals in aquatic biota, crayfish have sufficient tissues for a bioaccumulation survey. For this purpose, we examined the gills, muscles and hepatopancreas. We confirmed that the hepatopancreas was the primary target for accumulation of most of the examined elements (i.e., cadmium, chromium, copper, nickel, zinc). For higher relevance, crayfish surveys were compared to fish samples collected from the same locations.

The second part of this work particularly focused on water quality biomonitoring based on the evaluation of crayfish ethophysiological characteristics. We examined crayfish reactions to both chemical (i.e. chloramine, chlorides, nitrites) and natural odours (i.e., food, heterosexual conspecifics, predator, etc). This approach was shown to be simple yet at the same time, complex and efficient. Such monitoring technique is easily implemented and does not demand long, complicated analyses, since monitored parameters, locomotor and cardiac activity, are evaluated immediately in real time. However, one complication is related to the unpredictability of an animal's reactions. Because studied characteristics may often affect each other, they need to be carefully traced and interaction between measured characteristics needs to be eliminated. The usefulness of such biomonitoring is conditioned by a reliable combination of behaviour and physiology, which enables detection of complex animal responses to environmental changes.

As reported in the third part, we submitted an application for a patent of the developed system, and described in the patent sensor will be protected as utility model. Moreover, other crustaceans with sufficient carapace size (e.g., shrimps, crabs, molluscs) can be successfully investigated using presented system. The only challenge is that living organism can clearly indicate disruption of ambient conditions, but cannot detect what it has caused. However, there are powerful analytical techniques now, developed exactly for accurate determination of various compounds.

The heart rate is species- and conditions-specific, so it cannot be applied as unified measure for all crayfish species, while visual analysis of heartbeat primary curves can be useful for establishment of referent crayfish heart rate values at their different functional states. The final part of the thesis is devoted to this issue.

In conclusion, the developed biomonitoring system was shown to be highly practical unit using noninvasive technique for investigation of crayfish reactions under model conditions, with the potential of further application at broader research and industrial arenas.

CZECH SUMMARY

Využití raků jako bioindikátorů kvality vody

Iryna Kuklina

Ekologické postavení raků se během posledních desetiletí zásadně posunulo od vnímání raků jako senzitivních indikátorů vodního prostředí po dnešní rozšíření řady tolerantních druhů schopných přežívat v širokém rozpětí nepříznivých podmínek prostředí. I přes diskuze, zda je či není rak správný bioindikátor, jsou ale raci stále považováni za neodmyslitelnou a klíčovou součást vodního ekosystému. Bez ohledu na to, jak jsou jednotlivé druhy raků vnímavé ke kvalitě vodního prostředí, všechny druhy mohou být velmi dobře využity v různých studiích zaměřených na biomonitoring. Hlavním cílem dizertační práce bylo vyvinutí a inovace systému pro kontinuální monitorování kvality vody s využitím raků jako bioindikátorů. Tím, že nejsou raci tak vývojově složití ve srovnání s obratlovci (např. rybami), ale dostatečně komplexní ve srovnání s ostatními bezobratlými s pevnou tělesnou schránkou (např. mlži), reprezentují užitečný objekt pro biomonitoring s dobrou možností manipulace s relativně snadným získáním, analýzou a interpretací dat.

První část práce je věnována vyhodnocení raků jako vhodných bioindikátorů. Potvrdili jsme, že při zjišťování akumulace těžkých kovů ve vodním prostředí mohou raci poskytnout dostatečné množství vhodných tkání a orgánů. Pro tento účel jsme analyzovali žábra, svalovinu a hepatopancreas. Primárně se většina těžkých kovů akumuluje v hepatopankreatu (chrom, kadmium, měď, nikl, zinek). Pro větší relevanci výsledků biomonitoringu byla data porovnána se vzorky odebranými z ryb ze stejných nádrží.

Zvláštní pozornost je v práci (druhá část) věnována vyhodnocení etofyziologických charakteristik, pohybu a srdeční činnosti k vyhodnocení kvality vody. Testována byla reakce na chloramín, chloridy a dusitany a později i na různé přirozené podněty (pachy potravy, jedince jiného pohlaví, dravce atd.). Tento přístup se svým způsobem ukázal jako na jednu stranu jednoduchý, ale v zásadě komplexní a sofistikovaný. Jednoduchost tohoto přístupu spočívá v jeho rychlém využití a snadnějším vyhodnocení změn srdeční a pohybové aktivity přímo v reálném čase, oproti dlouhým a složitým analýzám. Jisté komplikace mohou souviset s nepředvídatelnou reakcí zvířat, protože se měřené parametry mohou často navzájem ovlivňovat, což musí být řádně sledováno a eliminováno. Jedinečnost našeho vyvinutého systému spočívá právě v kombinaci vyhodnocování chování a fyziologických parametrů, což poskytuje možnost relevantního vyhodnocení reakcí zvířat na změnu prostředí.

Na vyvinutý systém byla podána patentová přihláška a senzor bude ochráněn jako užitný vzor. Tento systém je využitelný i pro ostatní korýše s dostatečnou velikostí krunýře, stejně tak jako pro vodní mlže. Jedinou slabinou systému může být to, že živý organizmus je sice schopný přímé reakce na změnu prostředí, ale není schopný přesně specifikovat příčinu. Nicméně k tomuto účelu mohou být následně využity přesné chemické analytické metody schopné dnes detekovat široké spektrum látek.

Ačkoli je srdeční tep druhově specifický a závislý na kondici jedince, a nemůže být tudíž jednoduše aplikován na všechny druhy, může být vizuální analýza primárních křivek srdečního tepu využitelná pro stanovení referenčních hodnot pro určité fyziologické stavy raků. Tomuto problému je věnována závěrečná část práce.

Závěrem lze říci, že vyvinutý systém pro biomonitoring je velmi dobře využitelná neinvazní metoda ke studiu reakcí raků na specifické podněty v kontrolovaných podmínkách, s možností další aplikace ve více vědních oborech i praxi.

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LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS WITH IF

Kuklina, I., Kouba, A., Buřič, M., Horká, I., Ďuriš, Z., Kozák, P., 2014. Accumulation of Heavy Metals in Crayfish and Fish from Selected Czech Reservoirs, BioMed Research International, ID 306103.

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Kuklina, I., Císař, P., Pautsina, A., Kouba, A., Kozák, P., 2012. Continuous monitoring of crayfish heart activity based on non-invasive method. In: Book of abstracts from AQUA 2012, Global Aquaculture Securing Our Future, 1.–5. 9. 2012, Prague, Czech Republic, Poster 482.

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