

Article

Crayfish as Bioindicators for Monitoring ClO₂: A Case Study from a Brewery Water Treatment Facility

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Abstract: This study focuses on the use of crayfish as bioindicators in the water treatment process during operating conditions. The crayfish physiological responses to water disinfected with chlorine dioxide (ClO₂) was evaluated. Monitoring was conducted at the private commercial enterprise Protivín Brewery in Czech Republic under standard operating conditions. This brewery has a water treatment facility, where ClO₂ is used for water purification. A total of 25 adult signal crayfish (*Pacifastacus leniusculus*) were kept in separate flow-through aquaria receiving the purified water with ClO₂ concentrations ranging from 0.01 to 0.29 mg L⁻¹. Diurnal rhythms of 32% of crayfish was disturbed even at lower concentrations of ClO₂ (0.01–0.2 mg L⁻¹), while higher concentrations (>0.2 mg L⁻¹) affected all animals. A random decline and rise of heart rate was detected. In addition, the frequent occurrence of higher levels of ClO₂ significantly increased mortality. On average, mortality of crayfish occurred three to four weeks after stocking into the experimental system. Crayfish mortality is estimated to occur at concentrations exceeding 0.2 mg L⁻¹ of ClO₂. Our results suggest that long-term exposure to ClO₂ adversely affects crayfish physiology. In addition, the results of this study could contribute to the use of crayfish as bioindicators in long-term water quality monitoring under industrial conditions.

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

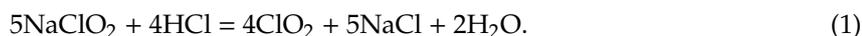
1. Introduction

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

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

While different compounds may be used for water purification, the most effective disinfectant is chlorine dioxide [12]. It is a powerful oxidant among chlorine compounds and it is widely applied

in surface water disinfection [13]. There are a few chemical reactions that produce chlorine dioxide (ClO_2), and one of them is the hydrochloric-acid-sodium-chlorite reaction [13]:



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

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

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

2. Materials and Methods

2.1. Monitoring Process

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

2.2. Monitoring System

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

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

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

2.3. Experimental Animals

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

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

Before monitoring commenced, the crayfish (with attached sensors) were acclimatized to lower temperatures in incubators (thermostatic cabinets Liebherr FK 5440, Liebherr-Hausgeräte Ochsenhausen GmbH, Ochsenhausen, Germany), where the temperature was decreased by 1 °C each following day. When acclimated temperature reached 10 °C, crayfish were transported in thermo-boxes in a small amount of water from the laboratory to the brewery by car (approximately 10 km). Before the experiment, crayfish were visually examined for absence of diseases and their biometrical measurements collected: Carapace length (mean ± SD): 43.8 ± 0.77 mm; total length: 90.13 ± 1.6 mm; and total weight: 33.68 ± 2.03 g. Weight and length was measured with digital calipers (Schut Geometrical Metrology, Groningen, The Netherlands) and an electronic balance (Kern & Sohn GmbH, Balingen, Germany). Both sexes of crayfish were used based on the previous study [23] which found no substantial differences between their reactions to stimuli. Only crayfish with intact appendages (antennae, chelae, and walking legs) were used in the experiment. During the experiment, crayfish were kept in separate 10 L flow-through aquariums, each receiving ClO₂-treated water with temperature of 10 ± 0.5 °C and pH 8.3 ± 0.5, under constant photoperiod 12:12 light-dark cycle. Each aquarium was provided with an artificial shelter (halved ceramic flower pot). A hole made on the upper surface of the shelter permitted recording of cardiac activity, even when crayfish (with the attached sensors) were inside the shelter. The experimental system could hold ten crayfish simultaneously. Five crayfish received a heart rate monitor each, while the other five crayfish were kept as reserves. In case of molting or mortality, an individual was replaced by one of the reserves. Thus, twenty-five animals were used in total throughout the monitoring period. Animals were fed daily with commercial food pellets (Sera GmbH; Heinsberg, Germany), and remains and feces were removed via daily syphoning.

2.4. Statistical Analysis

The data recorded from treated crayfish was divided between three groups in accordance with the day of exposure to maximum ClO₂ concentration (C_{\max} ; ClO₂ > 0.2 mg L⁻¹): Group one got C_{\max} on day 4 ± 2; Group two on day 13 ± 1; and Group three was exposed to C_{\max} on day 38 ± 6 after stocking to experimental aquarium system (Table 1). The data was grouped for subsequent analysis.

Table 1. Crayfish division based on the day of exposure to maximum concentration of ClO₂ (C_{\max}); life duration after C_{\max} treatment over the entire exposure period; and N—Number of crayfish in each group. Data presented as means ± SD.

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

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

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

3. Results

3.1. Ecdysis Period

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

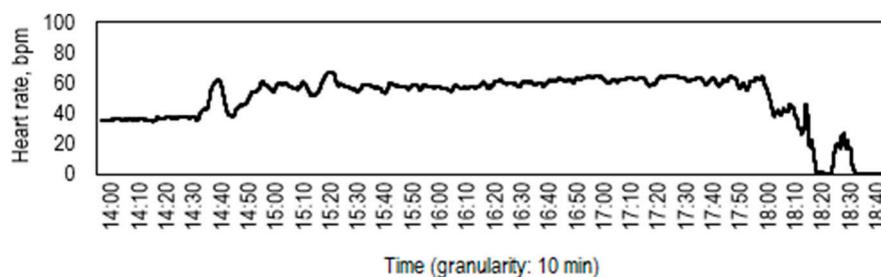


Figure 1. Heart rate of crayfish *P. leniusculus* four and half hours before molting. The fluctuating line shows heart rate, beats per minute (bpm).

3.2. Diurnal Rhythm

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

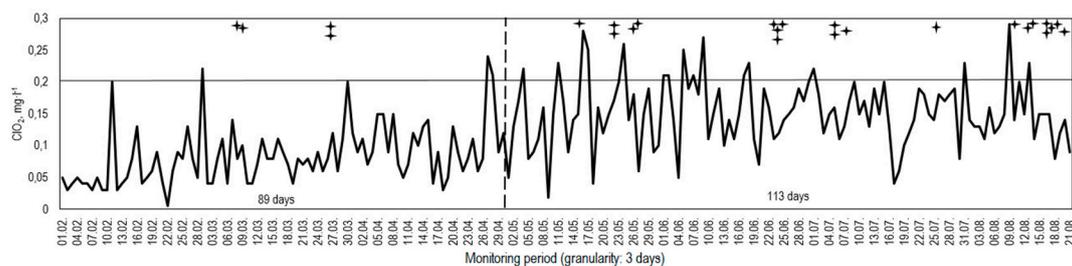


Figure 2. Chlorine dioxide (ClO_2) concentration and crayfish mortalities during the monitoring period. The stars (+) indicate crayfish mortalities; the fluctuating line indicates levels of ClO_2 concentration; the solid horizontal line indicates level of ClO_2 concentration 0.2 mg L^{-1} ; the punctuated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

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

Table 2. Average heart rate expressed as beats per minute of all monitored crayfish from the three groups during the ClO₂ exposure period. Mean ± SD.

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

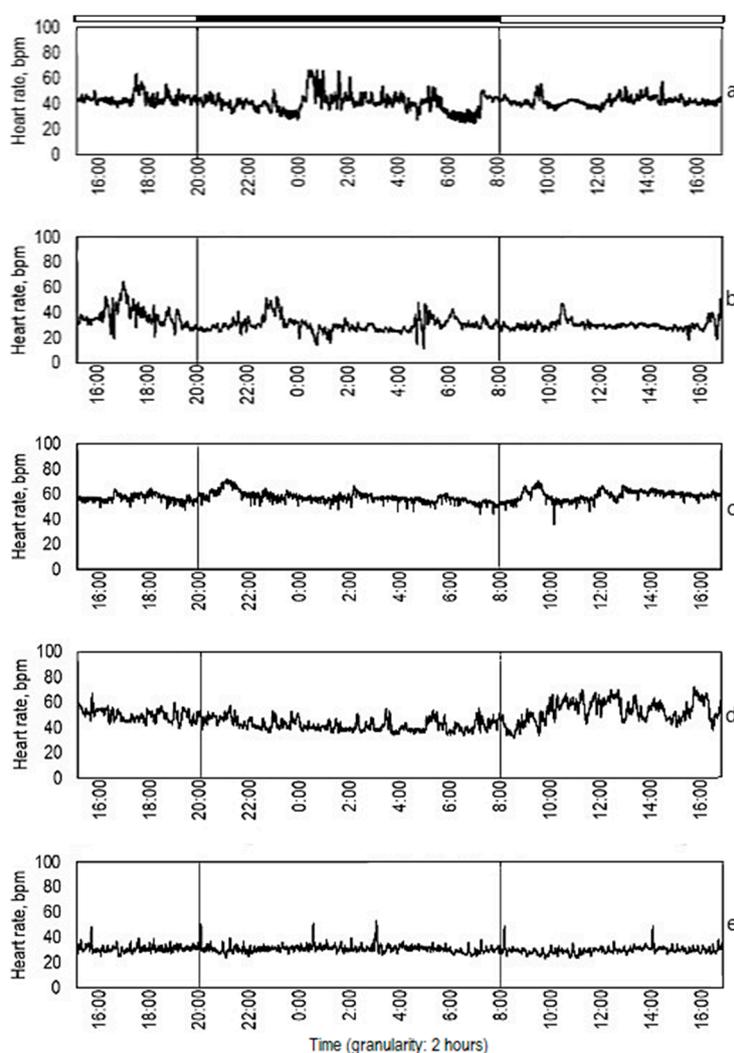


Figure 3. Examples of heart rate of five crayfish *P. leniusculus* during the day and night period, 5th to 6th of June 2017 with ClO₂ concentrations of 0.25 mg L⁻¹ and 0.19 mg L⁻¹, respectively. The fluctuating line indicates heart rate in beats per minute (bpm), while the two vertical lines distinguish night- and day-time.

3.3. Mortality

High ClO₂ concentrations (0.2–0.29 mg L⁻¹) disturbed the diurnal rhythm of all individuals, inducing loss of rhythmicity and subsequent mortality (Figure 4). Mortality increased along with more frequent occurrences of high ClO₂ concentrations. During the first period (89 days), where high ClO₂ concentrations (higher than 0.2 mg L⁻¹) were recorded five times, four crayfish died. During the second 113-day period, where high ClO₂ concentrations occurred 23 times, 21 crayfish died. Thus, in the first period mortalities occurred 5.3 times less than in the second one. No individual survived the experiment (Figure 4).

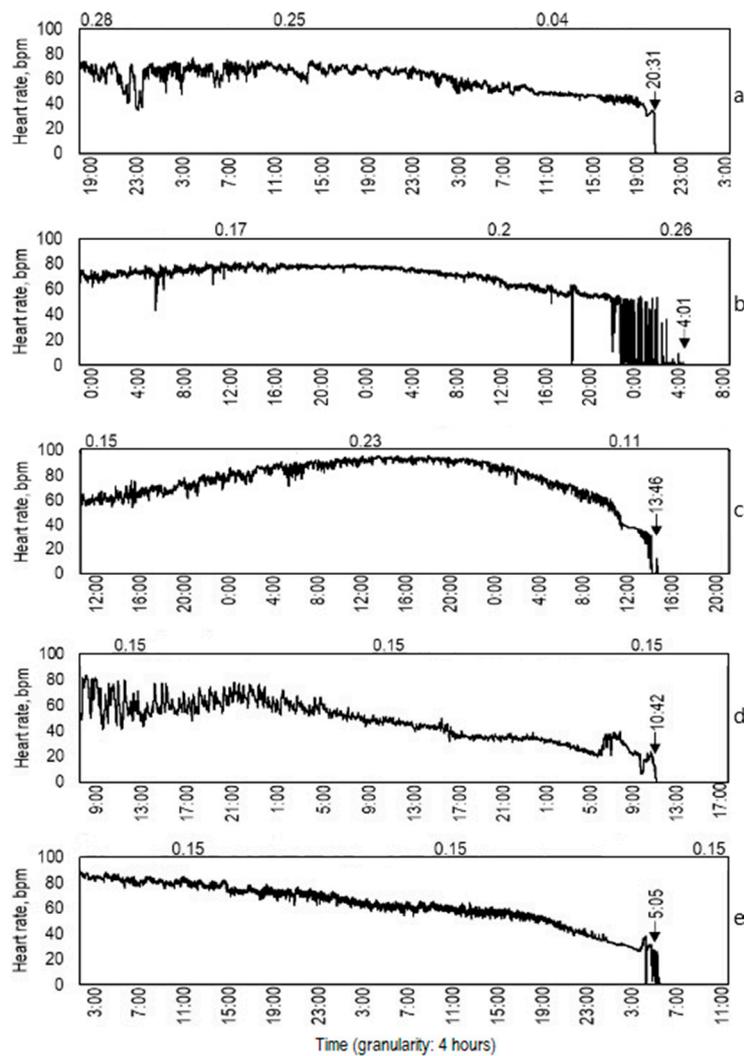


Figure 4. Examples of heart rate of five crayfish *P. leniusculus* two days before mortality occurred. The line indicates heart rate in beats per minute. Daily ClO_2 (mg L^{-1}) concentration are situated above the graphs, time of death is indicated by the vertical arrow.

Life Duration after Exposure to C_{max}

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

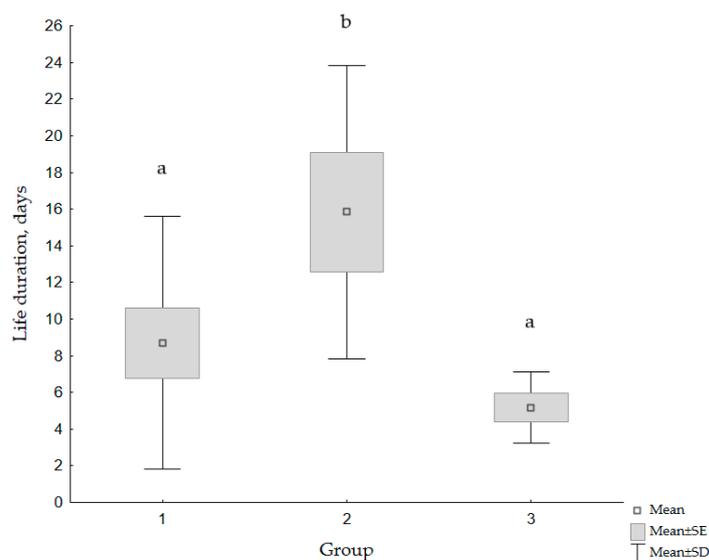


Figure 5. Life duration after C_{max} exposure of crayfish three experimental groups: Group one got C_{max} on day 4 ± 2 ; Group two on day 13 ± 1 ; and Group three was exposed to C_{max} on day 38 ± 6 . Life duration was: 9 ± 7 days, 16 ± 8 days, and 5 ± 2 days, respectively. There was a significant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

4. Discussion

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

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

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

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

Consequently, the ClO₂ used in our monitoring and heavy metals used by Styřishave et al. [7] and hydroquinone used in Kuznetsova et al. [21] can be toxic compounds at certain concentrations, and may negatively affect the health of aquatic organisms and even induce their mortality.

Not only are the loss of circadian rhythmicity suspected to induce crayfish mortality, but also the changes in physiology. The gills of fathead minnows *Pimephales promelas* were negatively affected by 0.13 mg L⁻¹ of ClO₂ concentration [25]. Chupani et al. [26] found heavy histopathological changes in crayfish exposed to peracetic acid (2–10 mg L⁻¹), while similar effects often induce mortality in juvenile grass carp *Ctenopharyngodon idella* [27] and channel catfish *Ictalurus punctatus* [28]. Subsequently, ClO₂ could induce adverse cardio-respiratory responses, reduce larval rainbow trout (*Oncorhynchus mykiss*) growth in concentration above 0.3 mg L⁻¹ [17] and cause oxidative damage and changes in antioxidant defenses in the heart tissue of juvenile rainbow trout [19]. Hence, it could have a similar effect in crayfish. Moreover, ClO₂ is more toxic to aquatic organisms than chlorite and peracetic acid [17,18]. Therefore, considering how ClO₂ is harmful for non-target aquatic animals and that it has higher toxicity than other substances, ClO₂ might likely have an adverse effect on crayfish tissues, leading to various disorders and subsequent mortality.

Peak concentrations of ClO₂ (0.2–0.29 mg L⁻¹) observed during our experiment significantly influenced the life duration of animals. Another study determined that 1–5 mg L⁻¹ of ClO₂ induces mortality of zebra mussel *D. polymorpha* [20].

When the C_{max} occurred, crayfish mortality was noticed after approximately 10 ± 7 days. Group one could likely not survive due to immediate exposure to increased ClO₂ concentrations, which resulted in rapid mortality. The prolonged exposure of Group three to low-to-medium concentrations of ClO₂ resulted in a cumulative effect, preventing organ and tissue regeneration, and resulted in crayfish mortalities 5 ± 2 days after C_{max} occurred. Group two, which was exposed to moderate ClO₂ concentrations within relatively short time (longer than Group one but shorter than Group three), had the longest life duration after getting C_{max}. This may suggest that crayfish responses differ between individuals.

5. Conclusions

Changes in crayfish heart rate and circadian rhythmicity could provide information about their functional state and help us make inferences on environmental state. Crayfish's physiological sensitivity allow early detection of increased levels of harmful chemicals, thereby presenting a practical solution for proactive water quality monitoring. Our results suggest that the changes in heart rate and diurnal rhythm of treated animals was crayfish-specific, which may stem from their varying functional state and individual physiological response to ClO₂ concentrations. There was a direct correlation between C_{max} and crayfish mortality. ClO₂ adversely affected crayfish circadian rhythm. In conclusion, this study demonstrated that crayfish could serve as effective bioindicators for long term practical water quality monitoring.

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

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