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Effects of Sublethal Exposure to Three Water Pollutants on Scototaxis in Rare Minnow (*Gobiocypris rarus*)

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Abstract: The biological early warning system with fish behavior as the detection index is an efficient and rapid early warning technology for the ecological damage caused by water pollutants. However, the attempt to apply the scototaxis (dark preference) behavior of fish to biological early warning is still relatively lacking. In this study, we delved into the dark and light preferences of the rare minnows (*Gobiocypris rarus*), employing three distinct tank configurations. Additionally, we systematically examined the modulating effects of environmental illumination, nutritional status, and the number of test subjects on this behavior, aiming to establish optimal experimental parameters for its observation. Furthermore, cadmium ions [Cd^{2+}], tricaine methanesulfonate [MS222], and *p*-chloroaniline were employed as representative heavy metal ions, neuroactive agents, and organic toxicants, respectively, to test the impact of chemicals on scototaxis in gradient concentrations. The results demonstrated that the rare minnow exhibited a clear scototaxis (dark preference), and this behavior was not affected by the nutritional status of the test fish, the illumination, or the number of subjects. While the dark chamber was consistently the preferred location of rare minnows during the chemical exposure tests, the degree of scototaxis by the rare minnow significantly decreased at $Cd^{2+} \geq 3$ mg/L, MS222 ≥ 11 mg/L, and *p*-chloroaniline ≥ 29 mg/L, suggesting a potential disruption of their innate behavioral patterns by these chemicals. These findings underscore the sensitivity of rare minnows to water pollutants. Therefore, the scototaxis behavior of rare minnows can be a potential and useful behavioral indicator for biological early warning, which can be used for early biological warning of sudden water pollution caused by chemicals such as Cd^{2+}, MS222, and *p*-chloroaniline.

Keywords: rare minnow; scototaxis; dark/light preference; biological early warning; effect of chemicals; water pollutants

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1. Introduction

Biological early warning systems (BEWSs) are water quality warning systems that utilize aquatic organisms for online monitoring. By detecting changes in biological physiology or behavior, BEWSs can rapidly alert in cases of water pollution or deterioration, thereby preventing sudden water pollution incidents [1]. Compared to the commonly used physicochemical analysis methods in traditional water pollution monitoring, BEWS technology can detect a wider range of toxic substances and effectively evaluate the comprehensive toxicity of different pollutants [2]. Fish are a commonly used indicator species in BEWS technology, and changes in their mortality rates, gene expression [3], hormone levels [4], proteins [5], and tissues and organs [6] under the influence of water pollutants have been widely employed as indicators in BEWSs to detect water pollution events. However, in practical applications, it has been found that the detection of these indicators involves varying degrees of complexity and time-consuming processes. In contrast, fish behavioral parameters are increasingly attracting the attention of researchers in the BEWSs field due to their simplicity and efficiency in detection. Fish behavior is highly sensitive to changes in water physicochemical factors, and variations in turbidity, dissolved oxygen levels, and the presence of chemical pollutants can elicit various behavioral responses, including excitement, sluggish swimming, and floating at the water surface [7,8]. Moreover, some complex behaviors such as color preference [9], swimming intensity [10], and taxis [11] can also be significantly impacted by chemical pollutants in the water. Studies have shown that behavioral indicators can efficiently detect sublethal concentrations of pollutants that are difficult to detect using mortality indicators, with a sensitivity 10 to 100 times higher than mortality indicators [12,13]. Additionally, behavioral indicators offer advantages such as shorter detection times, non-invasiveness, low damage, and scalability for repeated testing [14].

Scototaxis, defined as the preference for dark environments, is a widespread behavioral trait observed in numerous fish species, including zebrafish (*Danio rerio*), crucian carp (*Carassius langsdorfii*), Nile tilapias (*Oreochromis niloticus*), and guppies (*Poecilia reticulata*) [15,16]. Scototaxis is a behavioral response to novel environments. Staying in a favorable environment, such as a dark one, can help them find food, locate conspecifics, or avoid predators [17]. Studies have shown that the scototaxis of zebrafish is significantly influenced by factors such as light intensity [18], the number of subjects [19], and circadian rhythms [20]. Hence, the scototaxis displayed by fish emerges as an intriguing phenomenon that is intricately linked to environmental factors. Previous research on scototaxis predominantly employed it as an anxiety model, emphasizing anxiety testing paradigms and exploring the anxiolytic or anxiogenic properties of diverse pharmacological agents [21]. However, there is a notable absence of studies that have explored the potential of this behavior as an indicator in BEWSs. Some previous researchers have demonstrated that the scototaxis of fish undergoes pronounced alterations upon exposure to chemicals such as alarm substances [22] and alcohol [23]. Consequently, the scototaxis of fish may also serve as a promising indicator in BEWSs.

Most water pollutants consist of heavy metals and organic toxins, with some of the latter exhibiting neurotoxic effects. In the chemical exposure experiments of this study, cadmium ions (Cd^{2+}), MS-222 (3-Aminobenzoic acid ethyl ester methanesulfonate), and *p*-chloroaniline were selected as representatives of heavy metal ions, neurotoxicants, and organic toxins, respectively. Cd^{2+} is a pervasive environmental pollutant found in the atmosphere, water, and soil, posing significant hazards. It accumulates in aquatic organisms and crops [24]. Chloroanilines, including *p*-chloroaniline, are often associated with teratogenic, carcinogenic, and mutagenic effects. Due to their recalcitrance to biodegradation, they persist in the environment, posing threats to ecological systems and human health [25]. MS-222 is the only fish anesthetic approved by both the United States Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) [26]. The anesthetic effect of MS222 is achieved by disrupting the central nervous system of fish, making it an ideal representative of neurotoxicants.

The rare minnow (*Gobiocypris rarus*), a diminutive freshwater cyprinid endemic to the upper reaches of the Yangtze River in China, has garnered attention as the sole indigenous Chinese fish species recommended by "The Guidelines for the Testing of Chemicals" (State Environmental Protection Administration, SEPA), owing to its remarkable reproductive prowess, heightened chemical sensitivity, and other notable advantages [27]. As the development of fish behavior-based BEWS technology progresses, investigating the sensitivity of behavioral parameters of rare minnows to aquatic pollutants and their availability as BEWS indicators is crucial for enhancing the future development and application scope of this model organism. However, despite extensive research conducted over the past decade on the reproductive biology [28], aquatic habitat requirements [29,30], threpsology [31], genetics [32], and toxicology [33,34] of the rare minnow, there is a notable lack of studies on its behavioral characteristics, particularly scototaxis behavior and its alterations under chemical exposure. This study aims to bridge this gap by investigating the scototaxis patterns of the rare minnow and assessing the impact of experimental conditions on this behavior. Furthermore, Cd^{2+}, MS222, and *p*-chloroaniline were employed as representative heavy metal ions, neuroactive agents, and organic toxicants, respectively, to test the impact of chemicals on this behavior. We anticipate that our findings will enrich the behavioral characteristics of the rare minnow, evaluate the sensitivity of its scototaxis behavior to chemical exposure, and assess its potential in BEWSs, thereby providing a theoretical basis for the application of Gobiocypris rarus's behavioral parameters in BEWS systems.

2. Materials and Methods

2.1. Animals and Housing

The rare minnows used in this study were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. They were randomly selected from a closed colony, with an average body length of 2.02 ± 0.15 cm, and were in the juvenile stage, where their sex was undistinguishable. They were fed in 20 L transparent polycarbonate aquariums for more than 15 days. The aquariums were equipped with a circulating water system, and one-third of the water was changed every 3 days to keep the water clean. The photoperiod during the experimental period was artificially controlled to be 14 h of light and 10 h of darkness, from 08:00 to 22:00. The water temperature, pH, and dissolved oxygen levels were monitored daily using an HQ30d meter (Hach, Loveland, CO, USA) and maintained at 25–27 °C, 7.8–8.2, and 7.25–8.67 mg/L, respectively. The experimental fish were fed ozone-disinfected frozen red worms (Chironomidae flaviplumus larvae) (Yuerle, Tianjin, China) twice daily until satiation. The fish not involved in the chemical exposure experiments were recycled after the experiment was finished, and the fish involved in the chemical exposure were gathered and fed normally until death.

2.2. Chemicals

Stock solution of 2 g/L MS222 (3-Aminobenzoic acid ethyl ester methanesulfonate, purity > 99.5%, CAS NO. 886-86-2, WINYOUNG, Suzhou, China), 1 g/L Cd^{2+} ($CdCl_2 \cdot 2.5H_2O$, purity > 99.5%, CAS NO. 7790-78-5, SINOPHARM, Beijing, China), and 1 g/L *p*-Chloroaniline (purity > 98%, CAS NO. 106-47-8, Acros Organics, Newark, NJ, USA) were prepared 1 h before the tests, and all chemicals used in this study were of analytical grade.

2.3. Test Procedures and Apparatus

Transparent seamless glass tanks with dimensions of 30 cm × 15 cm × 20 cm were utilized for the experiments. The apparatus was placed within a gray stainless steel sink to mimic the normal breeding environment, and all other potential distractions were minimized. All experiments were conducted within the same time frame each day, from 9:00 to 18:00. In consideration of the welfare of the experimental animals and the accuracy of the experiment, the fish were fed to satiation before the experiment, and the fluorescent lamps at the top of the laboratory provided 500 lux illumination for the experimental environment (consistent with the daily feeding environment). Twenty fish were gently transferred to the test apparatus at a time and allowed to swim freely in aerated tap water at a temperature of 25–27 °C. Filming began after a 2 min period of adaptation. A video tracking system, consisting of a CCD camera and analysis software (TTQ, Shenzhen, China), was employed to track and record the location of each fish. A fish was considered to have entered an area if more than half of its body was within that area. To minimize any potential bias, the water was completely renewed, and the apparatus was rotated 180° clockwise after each test.

2.4. Scototaxis Test with Three Lighting Modes

Three tanks with distinct layouts and lighting modes were employed for this experiment, as illustrated in Figure 1. Tank 1 utilized a half-side mask to create differential lighting conditions. Tank 2 employed a single-point light source positioned at the side to generate a gradient of light. Tank 3 featured a two-color scheme, with black and white being used to provide contrasting visual cues. The number of fish entering the dark/light area was recorded every minute for a total duration of 30 min. The average value of the 30 recorded data points was calculated and used for subsequent statistical analysis. Each group underwent three repetitions, and no fish were reused in subsequent tests.

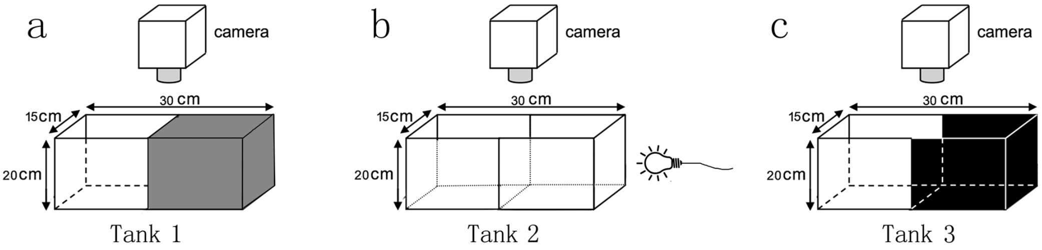

Figure 1. Experiment apparatus. Half of Tank 1 (three walls and the top) was surrounded by gray, opaque cardboard. A lamp at the top of the room provided 500 lux of light (**a**). Tank 2 was used in a completely dark environment, and a lamp was placed over one side of the tank to create a gradient of light. The average illumination of the chamber close to the lamp and the chamber far away was 500 lux and 50 lux, respectively (**b**). For Tank 3, the walls and floor of one chamber were painted black and the other white (**c**).

2.5. Environmental Factors Test

Tank 3 was used as apparatus in all subsequent experiments. In this experiment, the scototaxis test was conducted under the following conditions using a controlled variable method: (1) Two groups based on nutritional status: food-deprived and well-fed. The food-deprived group of fish was not fed for 24 h. (2) Four groups with varying levels of ambient illumination: 500, 1000, 1500, and 2000 lux. (3) Five groups with different numbers of subjects: 10, 20, 30, 40, and 50 fish. The rest of the procedures were similar to those in experiment 1, with one condition changed. The number of fish entering the dark/light area was recorded every minute for a total duration of 30 min. The average value of the 30 recorded data points was calculated and used for subsequent statistical analysis. Each group underwent three repetitions, and no fish were reused in subsequent tests.

2.6. Chemical Exposure Test

The aerated reconstituted water (5.5 $mg \cdot L^{-1}$ KCl; 294.0 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$; 123.3 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$; and 63.0 $mg \cdot L^{-1}$ $NaHCO_3$) was prepared with deionized water and analytical reagent in advance. According to the results of the previous acute toxicity exposure experiments on three chemicals with Gobiocypris rarus conducted by the team of authors, the 24 h median lethal concentrations (LC50) of the above three chemicals for rare minnow were 13.16 mg/L for Cd^{2+}, 45.10 mg/L for MS222, and 38.46 mg/L for *p*-Chloroaniline, respectively. The setting of concentration gradients in this study refers to the method of a previous report [35], where the LC_{50-24h} is taken as a concentration unit (TU), and 0.25 TU, 0.5 TU, 0.75 TU, and 1 TU are used as the experimental exposure gradients. The specific chemical concentration gradients set were 0, 3, 7, 10, and 13 mg/L for Cd^{2+}; 0, 11, 23, 34, and 45 mg/L for MS222; and 0, 10, 19, 29, and 38 mg/L for *p*-chloroaniline.

According to the results of 2.5, there was no significant difference in the scototaxis of rare minnow among the experimental groups with different nutritional status, illumination, and number of experimental subjects. Considering the welfare of the experimental animals and the accuracy of the experiment, the environmental parameters used in this experiment were consistent with those in 2.3, i.e., the experiment started after full feeding, the ambient illumination was 500 lux, and 20 fish were used each time. A total of 20 fish were transferred gently to Tank 3 before the experiment started. The film commenced following a five-minute adaptation period. The location of each fish was indicated every 1 min for a total duration of 60 min, which allowed enough time to establish full contact between the fish and the chemical solution. The average value of the 60 min recorded data was calculated and used for subsequent statistical analysis. Each group underwent three repetitions, and no fish were reused in subsequent tests.

2.7. Statistical Analysis

Data were checked for assumptions of normality and assumptions of homogeneity of variance by using the Kolmogorov–Smirnov and Levene tests. Wherever the assumption of normality was met, data were analyzed by One-way ANOVA and Bonferroni (multiple-comparison tests). If the assumption was not met, data were analyzed using the nonparametric Kruskal–Wallis test and Mann–Whitney U test (multiple-comparison tests). The statistical process and tests were performed using SPSS (version 19.0, IBM, Chicago, IL, USA) and Office Excel (version 2010, Microsoft, Redmond, WA, USA). Figures were created using Excel 2010, Originlab (version 8.0, Originlab, Northampton, MA, USA), and Adobe Photoshop (version CS6.0, Adobe, San Jose, CA, USA).

3. Results

3.1. Scototaxis Under Three Lighting Modes

According to the results presented in Figure 2, it is evident that the rare minnows showed a preference for the dark chamber over the light chamber across all three tanks ($p < 0.001$). There was a significant difference in the degree of scototaxis observed among the three tanks ($p = 0.008$). Specifically, in Tank 3, the number of fish entering the dark chamber (18.95 ± 0.41) was significantly higher compared to Tank 1 (15.01 ± 0.64, $p = 0.014$) and Tank 2 (13.42 ± 1.84, $p = 0.003$). However, there was no significant difference between Tank 1 and Tank 2 in terms of the number of fish entering the dark chamber ($p = 0.217$). The order of the degree of scototaxis among the three tanks was Tank 3 > Tank 1 > Tank 2.

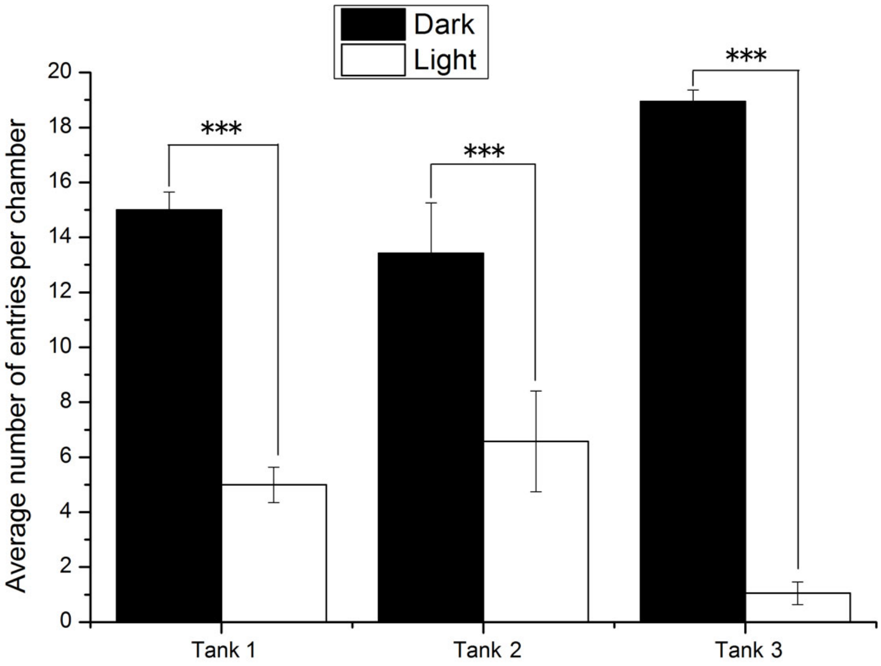

Figure 2. Quantitative distribution of rare minnow in two chambers in three apparatuses. Mean ± SD are shown; *** $p < 0.001$.

3.2. Scototaxis Under Different Environmental Conditions

In the experimental factors tested, the rare minnows showed a preference for the dark chamber over the light chamber across all experimental groups ($p < 0.001$). There were no significant differences observed in the degree of scototaxis based on different groups of nutritional status ($p = 0.348$, Figure 3a), illumination ($p = 0.247$, Figure 3b), and the number of subjects ($p = 0.194$, Figure 3c).

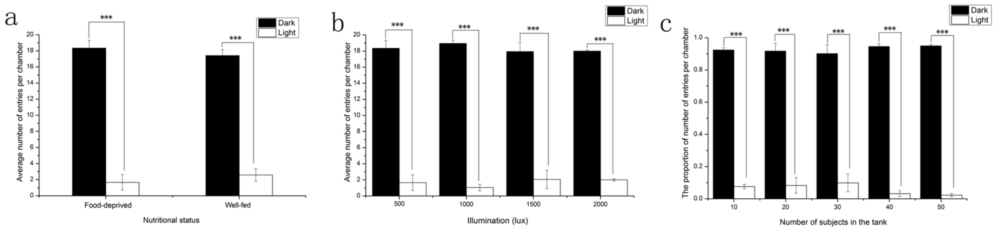

Figure 3. Quantitative distribution of rare minnow in two chambers with different nutritional status of test fish (**a**), illumination (**b**), and number of test fish (**c**). Mean ± SD are shown; *** $p < 0.001$.

3.3. Scototaxis Under Chemical Exposure

Compared with the control group, 29 mg/L and 38 mg/L of *p*-chloroaniline, as well as all concentrations of Cd^{2+} and MS222, significantly reduced the degree of scototaxis of the rare minnow ($p < 0.01$, Figure 4d,e). The lowest scototaxis degree was observed at a Cd^{2+} concentration of 7 mg/L (11.95 ± 1.10, $p = 0.001$, Figure 4d), an MS222 concentration of 34 mg/L (10.36 ± 0.35, $p < 0.001$, Figure 4e), and a *p*-chloroaniline concentration of 29 mg/L (14.31 ± 0.89, $p = 0.001$, Figure 4f). Despite the decreased degree, the scototaxis behavior of the rare minnow persisted at all concentrations of the three chemicals (Figure 4a–c), except for MS222 at 34 mg/L ($p = 0.104$, Figure 4b), indicating that they maintained their preference for the dark area.

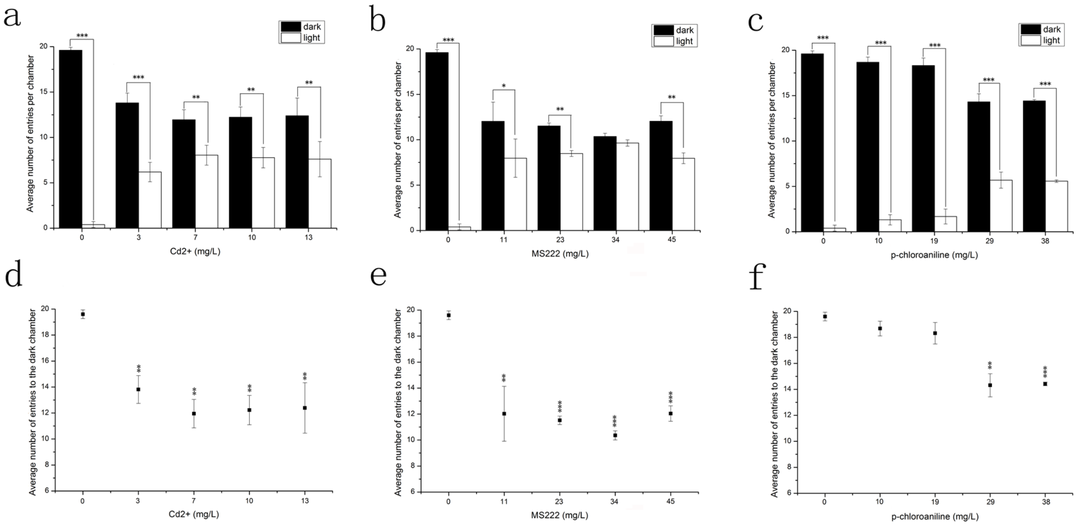

Figure 4. Quantitative distribution of rare minnow in two chambers (**a**–**c**) and average number of entries to dark chamber (**d**–**f**) upon exposure to Cd^{2+}, MS222, and *p*-chloroaniline. Mean ± SD are shown; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

Previous scholarly endeavors investigating zebrafish preferences for dark versus light chambers have yielded incongruent findings [18,22], with some research indicating a proclivity for darkness and others drawing contrasting conclusions [24,36]. Notably, variations in experimental setups have been identified as a contributing factor, with zebrafish displaying a tendency towards dark chambers in configurations characterized by uniform lighting but differing background hues and, conversely, favoring light chambers in scenarios where background color was consistent but illumination levels varied [16]. In the present study, we delved into the dark/light preference behavior of rare minnows, employing three distinct tanks with varied lighting modalities. Our findings unequivocally demonstrated that rare minnows exhibit a significant preference for dark chambers, irrespective of the prevailing lighting conditions. This preference for dimmer environments in rare minnows may be rooted in their natural habitat, typically occupying the middle-to-lower water strata, where light intensity is inherently reduced compared to the upper layers, where zebrafish predominate. Furthermore, the presence of dorsal melanophores in rare minnows serves as an adaptive mechanism, facilitating cryptic behavior by minimizing light refraction and reflection off their bodies, thereby decreasing their visibility to potential predators [37]. This physiological adaptation likely reinforces their innate tendency towards darker environments.

The relatively diminished contrast between the rare minnow and the gray/black backdrop within the tanks potentially augments their capacity for concealment, resulting in the heightened scototaxis preference observed in Tank 1 and Tank 3. Additionally, this study underscores the suitability of Tank 3 for conducting scototaxis assays, owing to its more pronounced and consistent inclination towards the dark chamber. Notably, the scototaxis exhibited by the rare minnow contrasts with the variability observed in zebrafish, displaying a relative stability and reduced sensitivity to environmental perturbations.

The results from Section 3.2 showed that the scototaxis behavior of the rare minnow has no significant difference when tested under different nutritional status, environmental illumination, and number of experimental subjects. Therefore, the influence of the above experimental parameters did not emerge as prominent factors influencing the scototaxis of the rare minnow. Consequently, rare minnows may be regarded as a potentially advantageous model organism for scototaxis research, offering a more reliable and consistent platform compared to zebrafish.

The scototaxis of rare minnows significantly waned as the concentrations of all three water pollutants examined in this study increased, with Cd^{2+} and MS222 eliciting a more pronounced effect than *p*-chloroaniline. As a heavy metal ion, Cd^{2+} imposes severe neurotoxicity, oxidative stress, and cellular damage upon exposure [38], compelling fish to evade contaminated waters and seek refuge in cleaner environments. This aversion intensifies under higher concentrations. The anesthetic properties of MS222, coupled with alterations in water quality (e.g., increased viscosity and foaming), also discomfort the fish, prompting them to flee. Conversely, although *p*-chloroaniline exhibits relatively weaker toxicity [39], its cumulative effects of toxicity and pungent odor disrupt the dark preference of rare minnows at elevated concentrations. This finding aligns with prior toxicology studies on rare minnows, which indicate that Cd^{2+} is more toxic than *p*-chloroaniline [39]. MS222 potently alters fish behavior, likely mediated through its anesthetic effect.

Collectively, the scototaxis of the rare minnow exhibits considerable sensitivity and prompt response to the exposure of three chemicals: Cd^{2+}, MS-222, and *p*-chloroaniline. Noticeable changes in this behavior occur within 30 min after exposure to these chemicals, demonstrating a concentration effect. Therefore, the scototaxis behavior of the rare minnow holds great potential as a novel behavioral indicator for application in BEWSs. Although this study utilized discrete observations every minute, in practical applications, the statistical interval could be reduced, or continuous monitoring could be implemented based on specific conditions to achieve more continuous statistical results and thus enhance observational accuracy.

When deploying BEWS indicators for early warning of sudden water pollution, two preset thresholds are typically established: a “warning point” for low-level contamination and an “alarm point” for high-level contamination. For the scototaxis of the rare minnow, a significant deviation ($p < 0.05$) in the number of fish entering the dark area from the normal behavior indicates that the chemicals or pollutants in the water have already impacted their normal behavior, serving as the “warning point” for early warning. On the other hand, a reversal in their scototaxis behavior (preferring the light area over the dark area) signifies that the chemicals or pollutants in the water have severely disrupted this behavioral pattern, causing the fish to lose its normal scototaxis, thereby functioning as the “alarm point”.

5. Conclusions

In conclusion, the results of this study indicate that the rare minnow exhibits pronounced scototaxis behavior, which shows strong stability under the influence of nutritional status, environmental illumination, and the number of experimental subjects, but is relatively sensitive to three environmental pollutants (Cd^{2+}, MS222, and *p*-chloroaniline). This suggests that the rare minnow is an ideal test organism for assessing scototaxis behavior, and its behavior holds promising applications in biological early warning.

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