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Original Article

Effects of Pretilachlor Herbicide on the Embryos and Adults of Javanese Medaka *(Oryzias javanicus)*

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Abstract: Pretilachlor (PRT), a commonly used herbicide in Malaysian rice fields, poses environmental concerns due to its potential impact on other living organisms. In this study, Javanese medaka (*Oryzias javanicus*) were used as a model organism to evaluate the effects of PRT on the embryo development and oxidative stress on adult species. Water samples from the rice fields that were treated with PRT were collected at four time points: 14 days before planting (DBP), 14, 42, and 70 days after planting (DAP). These samples provided a basis for preparing four different concentrations of PRT (0.040 mg/L, 0.045 mg/L, 0.050 mg/L, and 0.055 mg/L) for exposure experiments. Embryos exposed to these PRT concentrations exhibited teratogenic responses, including reduced hatchability and increased heart rates, even at the lowest concentration of 0.04 mg/L. The adverse effects were more pronounced at higher concentrations, with mortality rates reaching 60-70%. Observations revealed significant developmental disruptions, including early hatching and prolonged attachment to the yolk sac, which hindered the fry's ability to swim normally. These defects highlight the potential for PRT to cause substantial harm to aquatic life. In addition to embryo studies, adult Javanese medaka were also exposed to similar PRT concentrations. The results indicated that higher PRT levels led to increased oxidative stress, as evidenced by elevated metallothionein (MT) levels. Despite the increased stress, no mortality was observed in adult fish, suggesting that while PRT exposure is harmful, it may not be immediately lethal to adults. This study underscores the ecological risks associated with the use of PRT in agriculture, particularly its potential to contaminate water bodies and affect aquatic organisms. Continuous monitoring of PRT residues in local rice fields is crucial to ensure its regulated usage. The findings advocate stringent controls to mitigate the long-term ecological impacts of PRT, promoting safer agricultural practices to protect the aquatic environment.

Keywords: Pretilachlor, herbicide, Javanese medaka, developmental issues, toxicology study.

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1. Introduction

Chloroacetanilide herbicides, commonly detected in both groundwater and surface waters, present substantial risks to aquatic biota [1]. The increasing application of these herbicides has raised significant concerns about their contamination in aquatic ecosystems. Pretilachlor herbicide, $C_{17}H_{26}ClNO_2$ (PRT), a widely used chloroacetanilide herbicide in Malaysia, is employed as a selective pre- or post-emergence treatment in rice fields to manage broad-leaved weeds, grasses, and sedges [2]. PRT functions by inhibiting long-chain fatty acid synthesis and cell division, with dissipation processes including photodecomposition, volatilization, and microbial degradation [3]. Under field conditions, the half-life of PRT in soil is approximately 30.13 days [4], whereas in floodwater, it ranges from 0.87 to 1.52 days [5]. Chloroacetamide herbicides are recognized for their high toxicity, and some are considered carcinogenic [6]. In Malaysia, PRT residues have been detected at concentrations of up to 0.025-0.050 μg/L in the Muda irrigation scheme in Kedah, the country's largest rice granary [7]. Although residue levels fluctuate with application and planting seasons, concerns about their ecosystem impact persist. Elevated PRT levels have been reported to adversely affect bacteria, actinomycetes, fungi, nitrogen fixers, microbial biomass carbon [3], zooplankton species density [8], and freshwater fish [9].

Oryzias javanicus, also known as Javanese medaka, is a small, transparent fish extensively distributed in Malaysia, including Peninsular Malaysia and western Borneo, and in neighboring countries such as Thailand, Indonesia, and Singapore [10-12]. This species is available year-round and can be readily cultured under laboratory conditions, making it a popular choice for various ecotoxicological studies [13]. Javanese medaka is frequently utilized as an indicator species for detecting toxicants in water, including heavy metals and pesticides [13-16]. Due to its widespread distribution, abundance, and sensitivity, Javanese medaka is considered a crucial bio-indicator species for brackish and saltwater environments in tropical regions [11-14]. Despite its significance, there is a notable lack of literature regarding the toxicity of PRT on Javanese medaka.

2. Materials and Method

2.1. Sampling of PRT Herbicide in Rice Fields

Water samples were initially collected 14 days before planting (DBP) as controls, and subsequently at 14, 42, and 70 days after planting (DAP) following the application of PRT. The sampling technique involved creating small plots within the Kg Sg Leman rice planting area. Samples were collected from the center of these plots to ensure consistency and representativeness. A total of nine distinct sampling points were selected across the Kg Sg Leman rice fields. The precise locations of these points are depicted in Figure 1, which also includes the geographical coordinates for each sampling site to facilitate reproducibility and further study. At each of the designated locations, water samples were collected in triplicates from the surface layer of the canal. This method ensured that variations within each point were accounted for, thereby increasing the reliability of the data. The collected water samples were immediately transferred into 50 ml tubes, which were then wrapped with aluminum foil to minimize exposure to light and potential photodegradation of the herbicide residues. Following collection, the samples were promptly transported to the laboratory to prevent any alterations in chemical composition due to environmental factors. Upon arrival at the laboratory, the water samples were stored at a temperature of -20 °C until they were ready for extraction and analysis. This low-temperature storage was crucial for preserving the integrity of the samples and preventing any microbial activity that could affect the concentration of PRT residues.

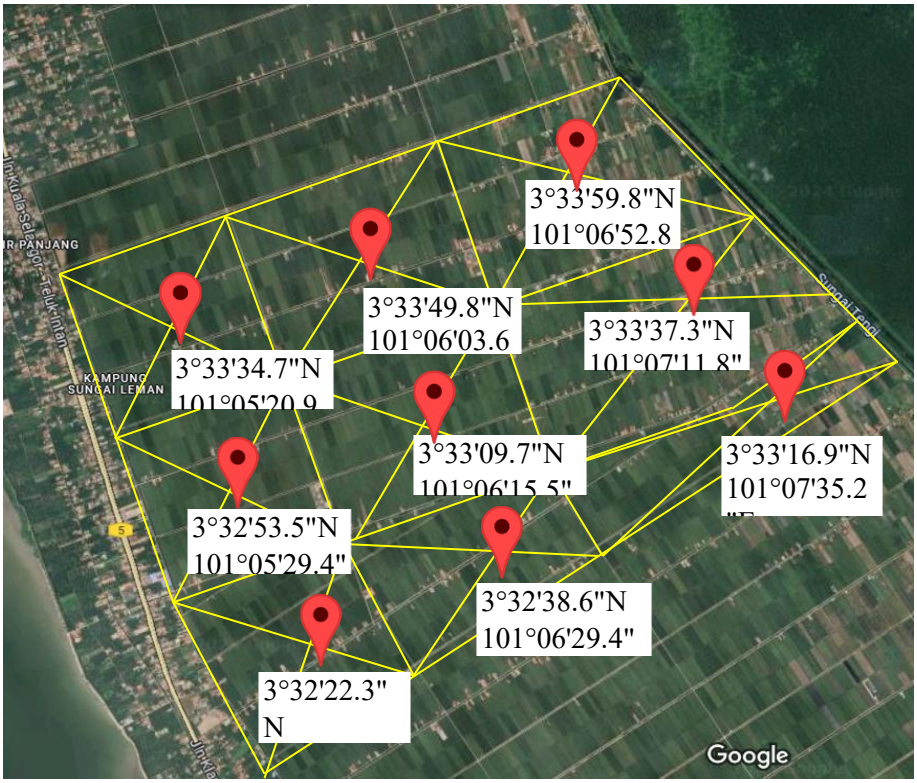


Figure 1. Site sampling of the study.

2.2. PRT Extraction Method

PRT with a purity of 99.0% was obtained from Sigma-Aldrich. High-performance liquid chromatography (HPLC) grade n-hexane and acetonitrile were sourced from Thermo-Scientific and Fisher Scientific, respectively. The separation of pretilachlor was conducted using a method adapted from RajaRajeswari et al., [4], employing a UV detector set to a wavelength of 210 nm. The mobile phase for the HPLC consisted of a mixture of acetonitrile and ultrapure water in a 90:10 ratio, with a flow rate maintained at 1 ml/min and a total run time of 10 min. For the quantification process, 20 µl of each sample was injected into the HPLC system. One milliliter of each water sample was transferred into a 2 ml micro tube and air-dried until complete evaporation of water. The residues were then reconstituted with 1 ml of n-hexane, followed by filtration through a 0.45 µm nylon filter to remove particulates. This prepared solution was subsequently injected into the HPLC for analysis. The experimental design employed was a Randomized Complete Block Design (RCBD), which was used to identify significant differences in PRT concentrations over the different sampling times. To analyze the data, SAS/STAT® version 13.1 software was utilized, providing robust statistical analysis tools necessary for evaluating the differences within the sampling times.

2.3. Exposure of PRT Herbicide on Javanese Medaka Embryos

The F1 generation of Javanese medaka was meticulously bred in the Department of Biology, Faculty of Science, Universiti Putra Malaysia. The adult breeding stock was collected from its natural brackish habitat in Kuala Linggi, Negeri Sembilan. To ensure their survival during transportation, the fish were placed in a container equipped with an air stone for

continuous oxygenation. The Javanese medaka is easily identifiable by its distinctive yellowish line on its fins and its nearly transparent body. Typically, this species is found in large schools on the water's surface. Upon arrival at the laboratory, the fish were acclimatized in incubating tanks set under ambient conditions until the pH levels stabilized at 6, with dissolved oxygen concentrations maintained between 6.0–8.0 mg/L. The Javanese medaka's exceptional adaptability to both freshwater and saltwater environments make it an ideal model organism for aquatic ecotoxicology studies [12, 13]. To facilitate breeding, the fish were subjected to a controlled light cycle of 14 h of light followed by 10 h of darkness and were fed artemia thrice daily [13, 14]. Fertilized eggs were carefully collected from the females each morning using a dropper and were subsequently surface sterilized in a solution containing 17 mM NaCl, 0.4 mM KCl, 0.36 mM $CaCl_2$, 0.6 mM $MgSO_4$, and 0.0002% methylene blue to prevent contamination. For the experiments, ten eggs were used per treatment, with each treatment replicated five times. The treatments involved using a commercially available PRT herbicide, with five concentration levels: T0 (Control), T1 (0.040 mg/L), T2 (0.045 mg/L), T3 (0.050 mg/L), and T4 (0.055 mg/L), reflecting the range of PRT levels typically found in rice fields. To maintain consistency in the experimental conditions, the treatment solutions were refreshed daily. For statistical analysis, a One-Way ANOVA followed by Fischer's Least Significant Difference (LSD) post-hoc test was performed to identify significant differences within the treatment groups. The data analysis was carried out using SAS/STAT® version 13.1 software.

2.4. Metallothionein Level in Adult Javanese Medaka

Adult Javanese medaka were exposed to the same concentrations of PRT as the embryos for a duration of 72 h. Each individual fish was placed in a container holding one liter of PRT solution, which was refreshed daily to ensure consistent concentration throughout the exposure period. Following the 72-h exposure, the adult fish were euthanized humanely and stored at a temperature of -80 °C until the extraction process. The extraction of Metallothionein (MT) protein from the fish tissues was carried out using the CUSABIO® Fish metallothionein ELISA Kit. The procedure began with homogenizing the samples using liquid nitrogen and a mortar and pestle to achieve a fine, consistent texture. An extraction buffer solution, comprising 0.5 M sucrose, 20 mM Tris-HCl (pH 8.6), 0.5 mM PMSF, and 0.01% β-mercaptoethanol, was then added to the homogenized samples. These samples were transferred into 2 ml tubes and centrifuged at 16,000 × g for 30 min at 4 °C to separate the supernatant. Fifty microliters of the supernatant were carefully pipetted into the microplate provided with the ELISA kit. To this, 50 µl of HRP-conjugate (1x) was added, and the microplate was shaken for 60 s to ensure thorough mixing. The plate was then incubated at 37 °C for 40 min to allow for binding reactions to occur. Post-incubation, 200 µl of wash buffer(25x)was added to each well, followed by aspiration of the buffer to remove unbound substances. This washing step was repeated five times to ensure cleanliness and accuracy. Following the final wash, the plate was lightly tapped on a clean tissue to remove any residual liquid. Subsequently, 70 µl of TMB substrate was added to each well under dark conditions, and the plate was incubated for an additional 20 min at 37 °C to develop the color reaction. Finally, 50 µl of stop solution was added to terminate the reaction, and the absorbance was read at a wavelength of 450 nm using a Rayto RT 2100C microplate reader. For the statistical analysis, a One-Way ANOVA was conducted, followed by Fischer's Least Significant Difference (LSD) post-hoc test, to determine significant differences between the treatment groups. This analysis was performed using SAS/STAT® version 13.1 software.

3. Results and Discussion

3.1 Standard Calibration for PRT

The standard calibration curve is crucial for calculating the amount of residue found in the water samples. Figure 2 depicts the calibration curve for PRT, described by the equation y = 2.9583x + 1.0758. To ensure the accuracy and reliability of the quantification, the coefficient of determination (R^2) must be as close to 1 as possible. For this technique, the R^2 value is 0.9205, which indicates a strong correlation between the measured data points and the regression line. This high R^2 value suggests that the data points are closely aligned with the regression line, thus providing reliable and consistent results for further analysis. The robustness of the calibration curve is essential for the accurate determination of PRT residues in environmental samples. The high R^2 value of 0.9205 confirms that the method is well-suited for quantification purposes, as it demonstrates a strong fit of the data to the calibration model. This ensures that the calculated concentrations of PRT residues are both precise and accurate, allowing for meaningful interpretation and comparison of the results across different samples and conditions.

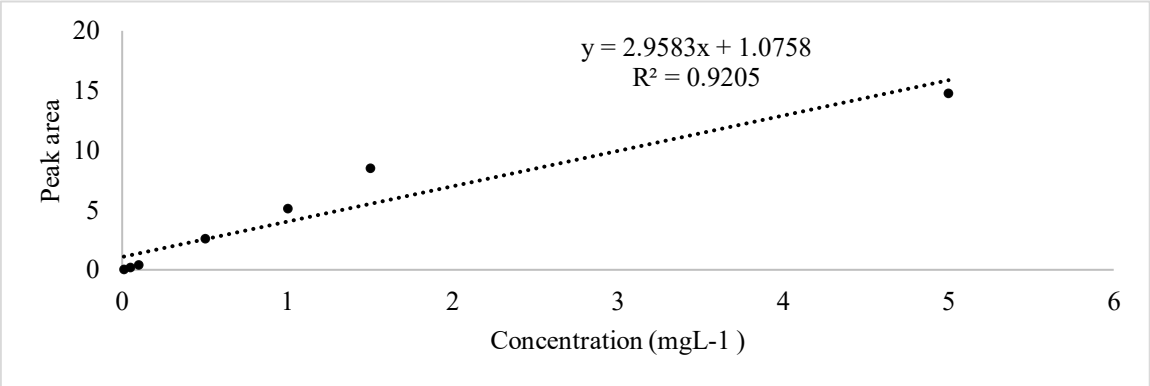


Figure 2. Standard calibration curve of PRT.

3.2. Recovery of PRT

Table 1. Recovery of PRT

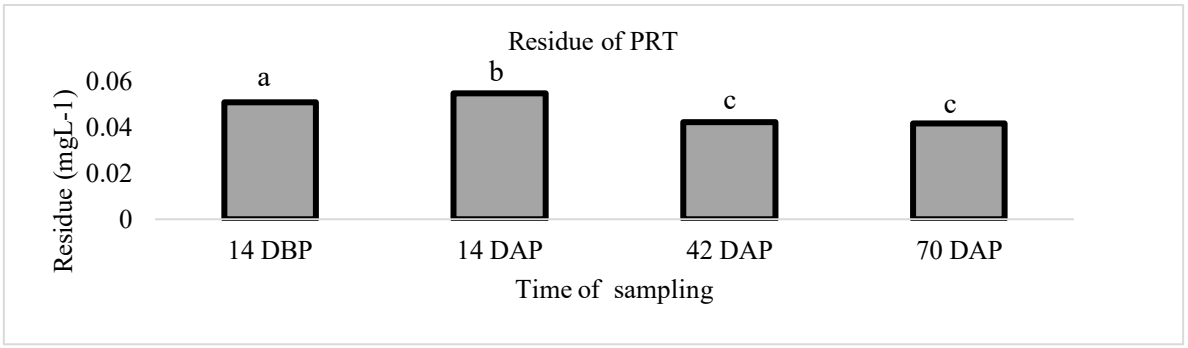
Targeted Concentration (mgL^{-1})	Recovery (%)
1.5	105.29±1.81
1.0	113.08±2.35
0.5	114.62±1.45

Spiking is a technique employed to assess the quality of an extraction procedure and to determine whether analyte detection is influenced by the sample matrix. In this study, the spiking technique was utilized to evaluate the accuracy and precision of PRT detection in water samples. The recovery rates of PRT were determined by adding known concentrations of PRT to the samples and measuring the extracted amounts. The results for PRT recovery were 105.29%, 113.08%, and 114.62% for concentrations of 1.5 mg/L, 1.0 mg/L, and 0.5 mg/L, respectively. These recovery values indicate that the extraction procedure is reliable and falls within the acceptable range of 80% to 120% [17], ensuring the validity of the analytical method used. These data are summarized in Table 1. The high recovery rates observed for all spiking concentrations confirm the robustness and efficiency of the extraction method, indicating that the sample matrix does not significantly interfere with the detection of PRT. This validation step is crucial in analytical chemistry to ensure that the method yields accurate and reproducible results, which are essential for meaningful environmental monitoring and assessment. The consistency of

the recovery percentages across different concentrations demonstrates the reliability of the technique in quantifying PRT residues in aquatic environments.

3.3. PRT Residue in Water Canals

Figure 3 presents the residue levels of PRT in the rice field over a specified period. Initially, the detected residue levels were 0.0509 mg/L at 14 days before planting (DBP). The levels increased gradually, reaching 0.0549 mg/L by 14 days after planting (DAP). Thereafter, a decline was observed, with residue levels decreasing to 0.0423 mg/L at 42 DAP and further to 0.0419 mg/L at 70 DAP. The significant differences in residue levels over time are indicated by different markers (a, b, c) in the figure. The analysis shows a significant difference between the residue levels, demonstrating that the changes in PRT concentrations were statistically significant at various sampling points. At 14 DAP, the residue levels were significantly higher compared to those at 14 DBP, illustrating the immediate impact of herbicide application. Subsequently, a marked difference was observed between the residue levels at 42 DAP and 70 DAP. This pattern is consistent with the expectations, given that the herbicide was applied early in the planting season. The high residues at 14 DBP and 14 DAP reflect the initial application, while the gradual decline observed at 42 and 70 DAP can be attributed to the short half-life of PRT and its dissipation processes, including photodecomposition, volatilization, and microbial degradation. This temporal variation in residue levels underscores the herbicide's persistence and its eventual breakdown in the environment.



*Means with different letters are significantly different
Figure 3. Residues of PRT in rice fields.

3.4. Hatchability

Figure 4 depicts the hatching rates of Javanese medaka embryos exposed to different concentrations of PRT. The data show a clear trend: hatchability percentages decrease as PRT concentration increases. Significant differences were observed between the control group (T0) and all treatment groups (T1 – T4). The most pronounced effect was noted in the T4 group, which exhibited the lowest hatching rate at 24%. However, no statistically significant differences were observed among the treatment groups (T1 – T4) themselves. The results indicate that even at the lowest dose tested, the presence of PRT in the environment can lead to over 50% fatalities in Javanese medaka embryos. This finding underscores the acute toxicity of PRT, as it induces fatal reactions in the exposed embryos. The data highlights the herbicide's potential threat to aquatic life, emphasizing the need for careful management and monitoring of its use in agricultural practices. These observations are crucial for understanding the broader ecological impacts of PRT contamination in aquatic ecosystems.

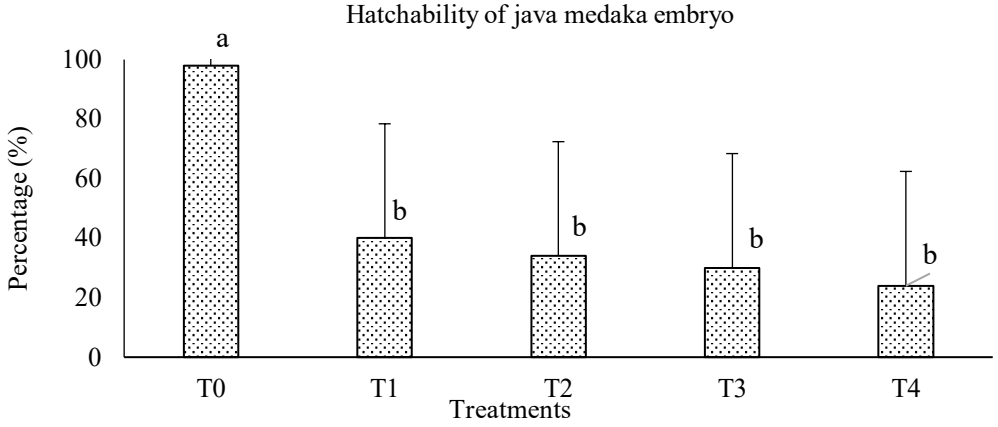


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Figure 4. Hatchability of Javanese medaka embryos.

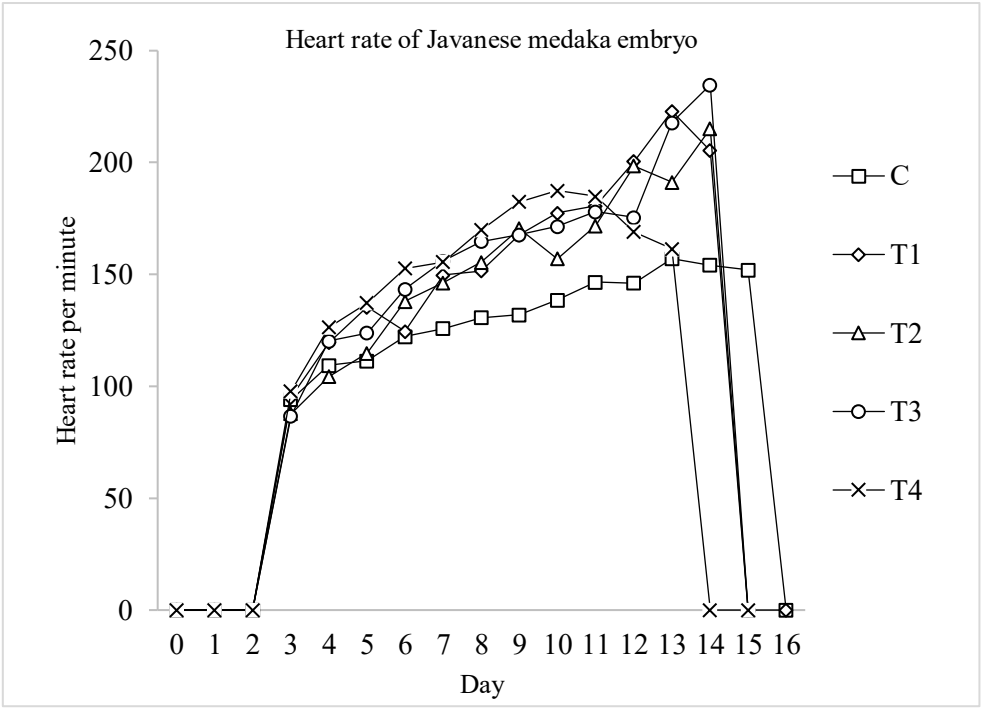

Figure 5. Heart rate of Javanese medaka embryo.

3.5. Heart Rate

The heart rate of Javanese medaka embryos was meticulously monitored over a period of 16 days as shown in Figure 5, during which the embryos hatched in the later stages of observation. For the control group (T0), the mean heart rate ranged from 94.02 to 157.04 beats per min. In stark contrast, embryos exposed to PRT at various concentrations (except for the highest concentration in T4) exhibited heart rates that exceeded a mean of 200 beats per min. Specifically, the highest recorded heart rates for the treatment groups were 223.00

beats per min for T1, 215.13 beats per min for T2, 234.75 beats per min for T3, and 187.50 beats per min for T4. These findings indicate that exposure to PRT, even at the lowest concentrations found in the environment, can elevate the heart rate of Javanese medaka embryos beyond their normal physiological levels. The elevated heart rates suggest a stress response or potential toxicity effect induced by herbicide. Additionally, there was a trend of increasing heart rates over time, correlating with the duration of exposure. Higher concentrations of PRT were also associated with a greater number of shorter incubation periods, suggesting that PRT exposure may accelerate the development process or increase stress, leading to premature hatching.

3.6. Developmental Defects

The majority of defects were observed when Javanese medaka embryos hatched prematurely, specifically between 7 and 9 days after fertilization. Normal hatching days for Javanese medaka eggs in freshwater are around 11 days [18]. In this study, hatching for control embryos can reach up to day 16th. A common defect was prolonged attachment to the yolk sac, where the fry was unable to detach until three days post-hatching. As illustrated in Table 2, at the PRT concentration of T1, 10% of embryos exhibited this defect, while at T2 and T3, the percentages were 6% and 4% respectively. No early hatching was detected at T4, which is likely attributable to the high mortality rates at this concentration. Furthermore, retarded growth was noted in 2% of the embryos, particularly at T4, the highest concentration of PRT. These embryos exhibited almost no growth until death occurred. Dead embryos were identified when no movements were observed and the embryos turned completely white. The various teratogenic effects observed in this study can be seen in Figure 6. Importantly, no defects were observed in the control group (T0), indicating that the observed defects were indeed induced by PRT exposure.

Table 2. Defect of Javanese medaka embryos

Defect/ Treatment	T0	T1	T2	T3	T4
Early hatch	0%	10%	6%	4%	0%
Retarded growth	0%	0%	0%	0%	2%

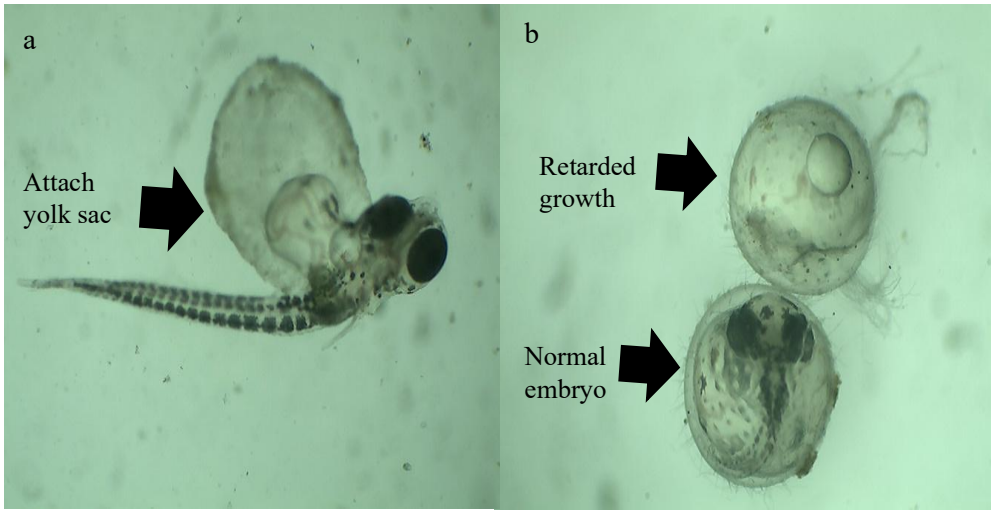

Figure 6. Images of Javanese medaka embryo. (a) Early hatch embryos with attach yolk sac and (b) developmental abnormalities embryo with retarded growth alongside a normal embryo.

3.7. Metallothionein Level

Figure 7 illustrates that MT levels in adult Javanese medaka increased in response to rising concentrations of PRT. The control group exhibited an MT level of 21.58 ± 1.25 pg/ml, which was significantly different from all PRT-treated groups. The highest MT level, observed in the PRT4 treatment group, was 46.44 ± 1.18 pg/ml. This level was significantly different ($p \leq 0.05$) from the MT level observed in the PRT1 treatment group, which was 36.245 ± 2.89 pg/ml.

These findings indicate that oxidative stress in the Javanese medaka escalated with higher PRT concentrations in their environment. The significant differences in MT levels, even at the lowest exposure levels, suggest that PRT induces oxidative stress in the fish. Despite the increased stress indicated by elevated MT levels, it was not lethal, as no mortality was recorded among the subjects during the study. This outcome highlights the resilience of Javanese medaka to oxidative stress up to a certain threshold and underscores the importance of monitoring sub-lethal stress markers in ecotoxicology studies.

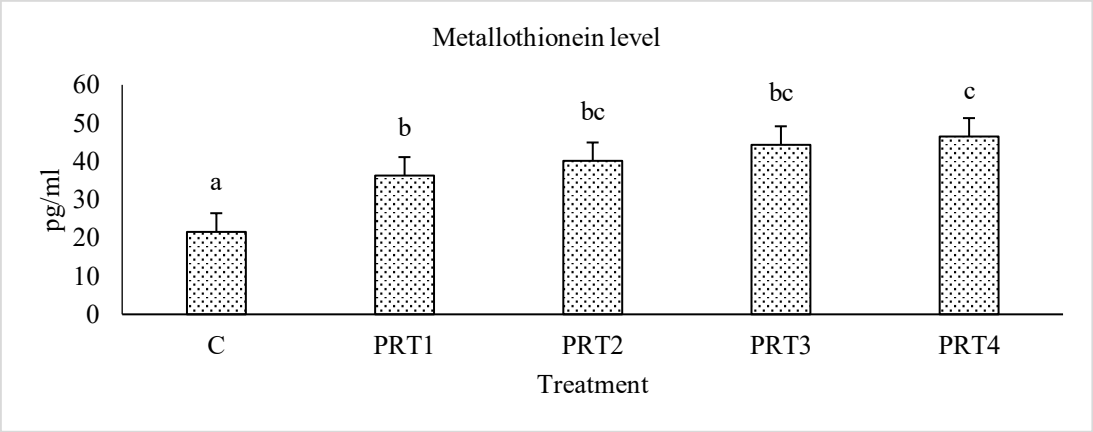

Figure 7. Metallothionein level in adult Javanese medaka following PRT exposure.

3.8. Discussion

The residue levels of PRT found in the water samples were consistent with those reported in other studies. Residue levels in other studies have shown significant variation, ranging from as low as 0.00084 to 0.01589 µg/ml [19] to as high as 0.936 to 1.233 µg/ml [20]. In Malaysia, one known study was found in the range of 0.025-0.050 µg/L [7]. In this study, the residue levels ranged between 0.0419 mg/L and 0.0549 mg/L, which falls within the reported local ranges. Residue levels of PRT decreased from 14 days after planting (DAP) to 70 DAP. PRT can persist in soil for up to 45 days post-application and has a strong tendency to adsorb clay loam soil [21, 22]. However, it has been documented that PRT can dissipate into paddy water, which explains the residues found in the water samples [21]. Its chemical stability allows it to accumulate in water and concentrate in aquatic organisms such as fish [23]. PRT residues have also been detected in drinking water, raising concerns due to their carcinogenic potential in humans [24, 25]. Furthermore, the persistence of PRT residues in soil can be influenced by various factors, including soil moisture, atmospheric carbon dioxide, temperature, and microbial activity [26, 27]. In summary, the residue levels of PRT in this study align with those found in other research, underscoring the importance of monitoring and managing pesticide residues to mitigate potential environmental and health risks. The toxicity of PRT to aquatic life is evident in this study, with Javanese medaka embryos exhibiting high

mortality rates - nearly 60% at the lowest concentration (0.040 mg/L) and over 70% at the highest concentration. Exposure to PRT also resulted in increased heart rates, a common symptom of toxicity, and oxidative stress, as observed in zebrafish embryos [28]. Teratogenic effects, although less common due to high mortality rates, were noted at a concentration of 0.055 mg/L, similar to findings in zebrafish [29]. Early hatching induced by PRT is detrimental, as it hampers the normal movement of Javanese medaka fry, exposing them to predators and contributing to their early deaths and population decline. Additionally, significant differences in MT levels in adult Javanese medaka were observed with higher PRT concentrations. MT is a cysteine-rich protein involved in detoxification and antioxidant defense. Elevated MT levels indicate increased oxidative stress in the organism. Similar increases in MT levels have been reported with exposure to other pollutants such as heavy metals [30-32]. Therefore, MT levels can serve as an indicator of pollution severity following exposure to hazardous chemicals, including herbicides. This study highlights the critical need for careful monitoring and regulation of PRT usage to protect aquatic ecosystems.

4. Conclusion

Exposure of Javanese medaka embryos to various concentrations of PRT herbicide, which mirrors the residue levels typically found in rice fields, resulted in pronounced teratogenic effects and high mortality rates among the embryos. These findings underscore significant concerns regarding the uncontrolled application of PRT due to its acute toxicity to aquatic life. The high mortality rates observed at even the lowest concentrations tested indicate that PRT poses a severe threat to the health and survival of aquatic organisms. Moreover, the teratogenic effects, which include developmental abnormalities, further highlight the potential long-term impacts on aquatic populations exposed to this herbicide. Given these findings, it is imperative to implement continuous and rigorous monitoring of PRT residues in local rice fields. This monitoring is crucial not only to safeguard the health of aquatic ecosystems but also to ensure that the application of PRT is conducted in a careful and judicious manner. By regularly assessing residue levels, stakeholders can better manage and mitigate the risks associated with PRT usage, thereby protecting both the environment and human health. Additionally, this study advocates for the adoption of integrated pest management strategies that minimize reliance on chemical herbicides like PRT and promote sustainable agricultural practices. The potential for PRT to bioaccumulate and cause adverse effects in aquatic organisms calls for stringent regulatory measures and increased awareness among farmers and policymakers about the environmental and health risks associated with herbicide use. Furthermore, research should continue to explore alternative, less harmful methods of weed control that can achieve agricultural goals without compromising the integrity of aquatic ecosystems. By prioritizing environmental health and sustainability, we can work towards a balance between agricultural productivity and ecological preservation.

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