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Research Article

THE EFFECT OF LEAD ION ON THE GROWTH AND SURVIVAL RATE OF *Oryzias curvinotus* FROM STAGE 1 TO 10 DAYS OLD

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ABSTRACT

Lead ion is one of the cations that affects the vitality, growth ability, causing skeletal deformities in fish development. This study was conducted to evaluate the effect of lead ion (Pb^{2+}) on 1 to 10-day-old Oryzias curvinotus larvae at different concentrations: 0 µg/L; 50 µg/L; 100 µg/L; 150 µg/L. The results revealed that: (i) the minimum Pb^{2+} concentration affecting the medak survival rate is 50 µg/L; (ii) the Pb^{2+} concentration significantly affecting the survival rate on the 5^{th} day is 150 µg/L. Based on the obtained results, we offered an equation to predict the survival rate of fish larvae and discovered the lethal LD_{50} concentration of Pb^{2+} after 10 days culturing is 98 µg/L and LT_{50} of Pb^{2+} at the concentration of 150 µg/L is 9,76 days. In conclusion, the higher concentration of Pb^{2+} causes the lower survival rate and the higher skeletal deformities of Oryzias curvinotus; the most effective concentration is 150 µg/L.

Keywords: ion Lead (Pb^{2+}); poisoned by heavy metal; larval fish; *Oryzias curvinotus*

1. Mở đầu

Nowadays, the development of social economy leads to the increase of water pollution which, both directly and indirectly, affects the humans' health. One the most harmful factors to aquatic environment is heavy metal. The amount of metal produced by human activities such as As, Pb, Cd, Cu, Ni and Zn are estimated to be released more than that of nautral metal sources, especially for Pb (Lead) which is 17 times higher (Kabata, 1995). Lead enters soil and water due to human impacts mainly by fertilizer, sewage sludge, pesticides and industrial production, which is an urgent problem (Ba, 2008; Muller, 2001). Lead not only has effects on aquatic environment but also affects directly animals living in it causing metabolic and nervous system disorder (Neal, 2011; Rice, 2011). Therefore, the early stage toxicology testings are recommended

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to evaluate the maximum acceptable toxic concentration and provide data for setting the water quality regulations (Ba, 2008). In Viet Nam, the influences of Pb^{2+} are evaluated primarily by physical or chemical methos, without the accurate evaluation on the growth of aquatic animals, especially on vertebrate animals. For that reason, in addition to evaluating heavy metal pollution directly by physicochemical methods, the use of fish as organisms indicate environmental pollution is being applied widely. The *Oryzias curvinotus* is a common species of freshwater fish in scientific research because of its widespread availability, small body, short life span for following easily and many practical meanings. Besides, the larval stages of fish are usually the sensitive one during their life span and the heavy metal accumulation in fish larvae affects the viability and the growth of them. Hence, this study basically evaluates the harmful effects of lead ion (Pb^{2+}) on the viability and deformation of the *Oryzias curvinotus* in larval stages (1-10 days old).

2. Material and Method

2.1. Material

Materials used for this experiments include:

- Glassware must be washed and steamed, sterilized and dried before using;
- Stereo and inverted microscope system with NIS Elements Fpackage version 3.2 attached;
- System of 4 aerators for aquariums;
- Algae used to raise larvae and fish are aerated 24/24 and eliminated the dead ones everyday;
- Fish feed includes commercial pellets. Besides, Bobo is also used as a type of fish feed;
- The *Oryzias curvinotus* fish in the larval stage (1-10 days old) were raised in the environment at different concentrations of lead contamination.

2.2. Method

2.2.1. Oryzias curvinotus maintenance and mating

- The *Oryzias curvinotus* fish, about 2 months old, were maintained at room temperature until getting sexual maturity.
- Arrange the aquariums tank in place with moderate light. Measure pH and water temperature 2 times/day. Monitor the room humidity and temperature regularly.
- Aquariums were equipped with aerators to provide enough oxygen for the fish. There were screens on the mouths of the tanks to prevent other animals from falling into the water or fish jumping out due to its fast swimming characteristics and the sensitivity to the outside environment. Algae were added to feed the larvae and create the fish farming environment. Change water 2 times/day.

- Until getting sexual maturity, males and females were kept separately. The glass mating tanks (60 mm diameter) were filled two-thirds full, with marbles at the bottom to make racks for embryos clinging. Males and females at the ratio of 1:2 were seperated by a transparent bulkhead in each tanks with a light-dark cycle condition right before mating. Then, remove the bulkhead for free mating to collect embryos. After about 5 minutes, the fish were transferred to the new tanks and the matting ones were left with the clinging-embryo marbles.

2.2.2. Examine and collect embryo and healthy fish larvae to arrange experiments

- Embryos were transferred to Petri dish by siphon. Under the stereo and inverted microscope system, examine the embyos' morphology and cell division in embryo. Only the embryos with good quality were used for experiment. The good embryos after being examined on Petri dish were put into 200ml beakers which were contaminated with lead at the following examined concentrations 0 µg/L; 50 µg/L; 100 µg/L until hatching into larvae.
- The *Oryzias curvinotus* in the embryonic stage and larvae stage was incubated at room temperature corresponding to the water temperature of 27-28^{0}C, with the same concentrations of lead ion with pH=7-7.5. Three days after the larvae hatching, feed them the plankton and the larvae found in the algae. Seven day larval fish were fed crushed bobo 3 times/day at 7 a.m, 12 p.m and 5p.m.

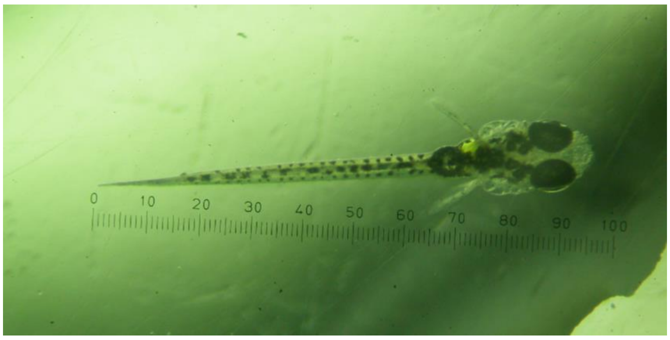


Figure 1. *The larvae of the Oryzias curvinotus were observed under the stereo microcope (X20)*

After the embryos hatched, select the healthy larvae to transfer them into the environment included Pb^{2+} at the corresponding examined concentrations: 50; 100, 150 µg/L and control (0 µg/L). The *Oryzias curvinotus* larval were observed daily (1-10 days old), record the number of live/ dead larvae and the changes of larvae in lead ion contaminated treatments day by day.

All of the larvae of the *Oryzias curvinotus* after hatching were observed under the stereo microcope (X20) to record abnormal morphologically development and from that the larval abnormalities were recorded at the surveyed concentrations. At each concentration, 15 larvae were observed, the experiment was then repeated 3 times.

2.3. Statistical Analysis

Data were analyzed based on a factor using MS Excel software to enter and process data. Average data are presented in the form of Average ± SD. Before conducting statistical analysis, the survival rate (%) was converted by the formula X_i=log (N_i)/N. Analyse the statistics by repeated one-way analysis of variance (ONE WAY ANOVA). The Duncan's method was used to compare the differences between the levels of experimental factors. The probability level $p < 0.05$ was accepted as the standards to evaluate that the differences had statistical meanings at 95% confidence. All of the statistical analyses were performed using the IBM SPSS Statistics version 19.0" software.

3. Results and discussion

3.1. Effects of lead ion on the survival rate at the larval stage (1-10 days old).

The survival rate is a reliable indicator of assessing the resistance of larvae (Wonmongkol, 2017). In this study, the variation in the survival rate of the larval *Oryzias curvinotus* at different concentrations of Pb^{2+} over the 10 day was recorded and presented in Table 1.

Table 1. *Survival rate (%) of larval fish stage from 6-10 days old at Pb^{2+} concentrations*

concentrations / Time	0 µg/L	50 µg/L	100 µg/L	150 µg/L	Average±SD
Day 1	100 ± 0.00^{ax}	100 ± 0.00^{ax}	100 ± 0.00^{ax}	97.78 ± 3.85^{ax}	99.44 ± 1.92^{a}
Day 2	100 ± 0.00^{ax}	100 ± 0.00^{ax}	100 ± 0.00^{ax}	97.78 ± 3.85^{ax}	99.44 ± 1.92^{a}
Day 3	100 ± 0.00^{ax}	100 ± 0.00^{ax}	95.56 ± 3.85^{abx}	93.33 ± 6.67^{ax}	97.22 ± 44.46^{a}
Day 4	100 ± 0.00^{ax}	95.56 ± 7.70^{abx}	95.56 ± 3.85^{abx}	91.11 ± 3.85^{ax}	95.56 ± 5.19^{ab}
Day 5	100 ± 0.00^{ax}	91.11 ± 7.70^{abcxy}	88.89 ± 3.85^{abyz}	80.00 ± 6.67^{bz}	90.00 ± 8.76^{bc}
Day 6	100 ± 0.00^{ax}	84.44 ± 10.18^{abcy}	82.22 ± 3.85^{by}	77.78 ± 7.70^{by}	86.11 ± 10.43^{cd}
Day 7	95.56 ± 3.85^{abx}	80.00 ± 13.33^{bcy}	80.00 ± 0.00^{by}	66.67 ± 6.67^{cy}	80.56 ± 12.54^{de}
Day 8	95.56 ± 7.70^{abx}	75.56 ± 13.88^{cdxy}	64.44 ± 13.88^{cy}	62.22 ± 3.85^{cy}	74.44 ± 16.53^{de}
Day 9	88.89 ± 10.18^{bcx}	64.44 ± 10.18^{dey}	62.22 ± 16.78^{cy}	57.78 ± 3.85^{cy}	68.33 ± 15.86^{ef}
Day 10	84.44 ± 7.70^{cx}	60.00 ± 6.67^{ey}	46.67 ± 13.33^{dy}	44.44 ± 10.18^{dy}	58.89 ± 18.6^{f}
Average±SD	96.44 ± 2.69^{x}	85.11 ± 6.01^{xy}	81.56 ± 4.34^{y}	76.89 ± 4.02^{z}	

a,b,c,d,e,f: show differences by line at 95% confidence (p<0.05)

x,y,z: show differences by row at 95% confidence 95% (p<0.05)

Based on the results of Table 1, it can be seen that the fluctuations of survival rate in the control treatment decreased through days. At the same time, during a fish's life, the larval stage is more sensitive than the adult one (Arufe, 2004). In newly hatched larvae, the bodies still carried the yolk-sac containing nutrients for themselves, however, when the the larvae were about 6 days old, the yolk-sac gradually exhausted and until the 10th day, the yolk-sac disappeared completely. At this time, the larvae must be self-feeding by finding external sources of food (such as plankton in algae). During the newly hatched phase, the survival rate of embryos was 44.44-84.44% lower than that of the embryonic stage because the larvae had certain changes in life. The larvae were not only no protected by the embryo' membrane but also exposed directly to the environment contaminated with lead ion, the larvae were not able to adapt yet, some organs had not been completed whereas the whole bodies had to be exposed to toxic substances such as lead ion, which made the survival rate at this stage lower than the embryonic stage.

Table 2. *Regression equation of survival rate of larval fish stage from 6-10 days old) at Pb^{2+} concentrations*

Predictive factor	**Estimated coefficient**	**SE value**	**P value**	R^2
Constant (α)	-0.039	0.06	0.008	0.927
Day (β)	1.242	0.051	0.00	
Regression equation of average survival rate by day (from 6 to 10 days old)	Y= -0.039x+1.242 (p=0.008)			

As shown in Table 1, the survival rate of larvae in control group changed during the cultured days. Specifically, from the 1st day to the 6th day, the number of larvae in control group did not change (p <0,05) because the larvae were hatching of the embryos chorion, the original viability was still good. After hatching, by the day 7th, the survival rate averagely decreased linearly by day. To be specific, the survival rate of larvae derceased by 15.56 % (from 100% on day 6 to 84.44% on day 10, shown in Table 1). The causes can be explained as following: on the first few days, the newly hatched larvae exposed directly to the environment contaminated with lead ion, vitality of the larvae from embyo still maintained, lead ion was not able to penetrate savagely to the bodies to harmful effects. The larvae lived based largely on the storage of the yolk sac until they started to let the external food feed them. The yolk-sac gradually disappeared on the day 6 to day 7. As a result, from day 7, the larvae had to adapt to a new self-feeding behavior so they became more sensitive and hence, the number of dead larvae at this stage was higher than that at stage 1-6 day.

In addition, fish from embryonic stage to larval one, it means that fish started to expose to the new environment, especially the one that is contaminated with poisons.

Based on the results of Table 1, we evaluated the fluctuation of the survival rate of the *Oryzias curvinotus* larval over the cultured days with the examined concentration of Pb^{2+}. In the surveyed treatments with different concentration of Pb^{2+}, the higher the concentration was, the sooner the time when fish had susceptibility and died was, to be specific, the concentration of 50µg/L was on the 4 th day (95.56±7,70%), the concentration of 100µg/L was on the 3th day (95.56±3,85%), the concentration of 150µg/L was on the 1st day (97.78±3.85%). In the surveyed treatments with the concentration of Pb^{2+}, at the concentration of 50µg/L, the 7th day is the time which had the highest rate of dead larvae (80.00±13.33%), at the concentration of 100µg/L it was on the 6th day (82.22±3.85%), at the concentration of 150µg/L it was on the 5th day (80.00±6,67%), this reduction is statistically significant ($p<0.05$). Thus, the higher the lead concentration in the treatments was, the sooner the time when the highest rate of newly hatched larvae started to be susceptible and die was. The experimental result is completely consistent with those of Barbar Jezierska (2002), which was proved that the newly hatched stage is the most sensitive stage of the larvae, the sensitivities of the newly hatched larvae increase because of the heavy metal contaminated environment's disadvantage such as lead ion. Larvae usually lack gill while permeable skin allows respiration and ion transport. Therefore, the toxins penetrate through skin. According to Peterson et al (2010) there are 30 genes involving to the development of the fish's body which were mutated during the Pb2+ exposure time.

Based on the results of Table 1, it is estimated that the lethal time variation of 50% the larval *Oryzias curvinotus* in different lead concentration ((LT_{50}) **(**Table 3**)**.

Table 3. *The lethal time variation of 50% the larval Oryzias curvinotus in different lead concentration ((*LT_{50}*)*

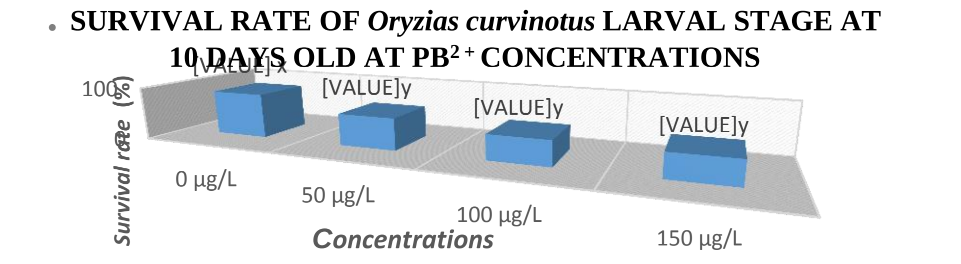
Concentration	**Estimated coefficient**	**The lethal time variation of 50% the larval (LT_{50}) (day)**
0µg/L	a=-0.015 (p=0.000) b= 1.049 (p=0.003) R^2=0.69	36.6
50µg/L	a=-0.048 (p=0.000) b= 1.113 (p=0.000) R^2=0.94	12.77
100µg/L	a=-0.058 (p=0.000) b= 1.133 (p=0.000) R^2=0.91	10.91
150µg/L	a=-0.064 (p=0.000) b= 1.118 (p=0.003) R^2=0.96	9.65

According to the results of Table 3, the higher the lead ion concentrations were, the quicker the time was when 50% of the *Oryzias curvinotus* died, from 12,77 days with the 50µg/L lead contaminated treatment to 9.65 days with the 150µg/L treatment.

In the early stages when being exposed directly to the lead contaminated environment, the organs of larvae started to have deformities. The organ system might become sensitive to the effects of toxins at certain stages during the early development but once developed they may no longer be too vulnerable to be affected (Ozoh, 1979). These experimental results are consistent with that of Vinodhini, Narayanan (2008), they said that when being poisoned with crom, niken, cadmium and lead ion, the *Cyprinus carpio* have 32 days to overcome the acute toxicity and increase the survival ability. Some of the toxic effects of the heavy metals on fish and aquatic invertebrates are the decrease in the growth, the increase of the abnormal growth, the decrease of the larval survival rate, especially at the time when the larvae start to eat the external food which may be poisoned. However, the newly hatched larvae do not usually have gill which is one of the most important organs to eliminate the poison.

When the *Oryzias curvinotus* larvae experienced most of the stages from the embryonic to the larval stages in the lead contaminated concentrations, they started to have stress. The stress response might lose its adaptive values and become functional disorders, which can lead to the growth inhibition, decreased reproduction, and reduced resistance to toxic.

It can be seen from Table 1, from the 1st day to the 4th day, the fluctuation of the survival rate of larvae decreased slightly when the lead concentration increased, nevertheless, the difference is not statistically significant ($P>0.05$). The reasons are that from the 1st day to the 4th day, the larvae the newly hatched larvae exposed directly to the environment contaminated with lead ion, the embryo viability was remained, lead ion was not able to penetrate savagely to the bodies causing harmful effects. The larvae were just out of the embryos with the chorion so the original viability was still good. From the 5th day to the 10th day, the increase in the lead concentration fluctuated the survival rate compared to that of the 1st day, the higher the lead concentration was, the more the larvae died (especially on the 5th, 7th and 10th day). After 10 days, the survival rate of larvae at the treatment 50µg/L was 60.00±6.67% and at the treatment 150µg/L the survival rate was 44.44±10.18%, this difference is statistically significant ($p<0.05$).



	0 µg/L	50 µg/L	100 µg/L	150 µg/L
Series1	84.44	60	46.67	44.44

Figure 2. *Survival rate of Oryzias curvinotus larval stage at 10 days old at* Pb^{2+} *concentrations*

Based on the regression equation of the survival rate of larvae over 10 days, it can be seen that the LD_{50} can be calculated Y=0.794-0.03X (R= 0.872).

Consequently, at the end of the experiment on the 10th day, the concentration for killing 50% of the larve was calculated by this equation LD_{50}=98 µg/L. According to WHO standards (1998) based on LD_{50} of the lead ion poisoning *Oryzias curvinotus* at the larval stage, it could be seen that the lead ion is extremely toxic to the fish at this stage.

Many authors have shown that heavy metals reduce the viability and the growth of the larvae, they also cause abnormal behavior or have effects on the structure of the larvae (mostly on spine deformation) (Khayatzadeh, 2010). In 1979, Ozoh reported the disappearance or tail deformation of lead ion poisoning fish and the deformations recored in this study were consistent with the work of fish deformation in lead ion contaminated environment.

3.1.1. Effects of lead ion on the deformed rate at the larval stage (1-10 days old)

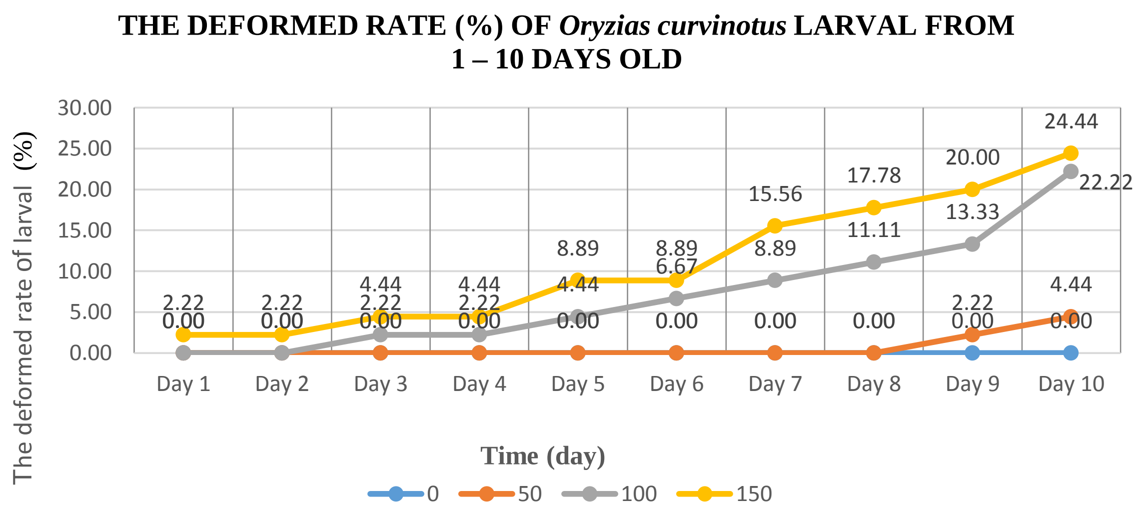


Figure 3. *The deformed rate (%) of Oryzias curvinotus larvae from 1-10 days old*

The most commonly observed deformation during larval development is spine deformation due to the lack in the vertebrae; it is because the newly formed vertebrae lose their charateristic V-shape and are assembled in a messy way. Hence, the spine formation is deformed and has the imperfections in the process of forming axes and spinal cord (which are important for the formation of vertebrae and central nervous system), which are consistent with Ozoh's research (1979).

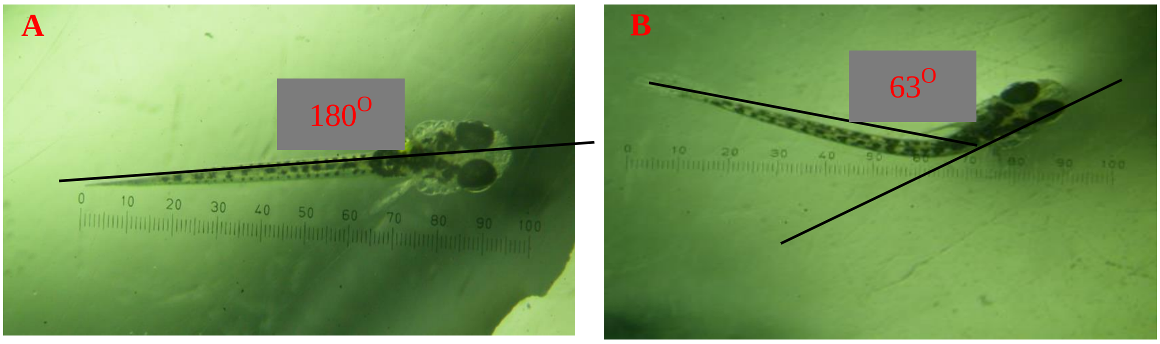


Figure 4. *A. Normal larvae (body axis is 180^{0}); B. Larvae with spine deformities (body axis is smaller than 180^{0}) (x20)*

In control group, no deformation was found. However, the survival rate of larvae decreased from the 7^{th} day to the 10th (from the rate of 95.56% to 84.44%).

At the concentration of 50 µg/L, the deformity appeared on the 9^{th} day (accounted for 2.22%) and on the 10^{th} day there was one more deformed larva, which made the rate of deformed larvae increase from 2.22% to 4.44%. Besides, the survival rate of normal larvae also went down gradually from the 3^{rd} day to the 10^{th} (from the rate of 100% to 60%).

At the concentration of 100 µg/L, the deformity appeared on the 3^{rd} day (accounted for 2.22%) but the 4^{th} day, the larvae carrying this deformity died, and there was no deformity appearing during the day. By day 5, the new deformities started to appear and exist until day 10. Also, on the 10^{th} day, other larvae had new deformities, which increased the rate of deformed larvae from 2.22% to 8.89%.

At the concentration of 150 µg/L, deformities appeared on the first day (accounted for 2.22%). Yet, until the 2^{nd} day, deformed larvae died. On the 3^{rd} day, deformed larvae continued to appear (accounted for 2.22%) and they died on next day. On the 5^{th} day, deformities then appeared (accounted for 4.44%) and that deformed larvae died on the 6th day. By the 7^{th} day, deformities appeared (accounted for 4.44%) and existed until the 10th day (accounted for 4.44%).

Therefore, the deformities of the larval *Oryzias curvinotus* appeared because of Pb^{2+} at the concentration of 50 µg/L (day 9) and the higher the concentration was, the sooner the time when the deformitties appeared was (day 3 at the concentration of 100 and day 1 at the concentration of 150 µg/L). At the concentration of 100 µg/L, larvae which had deformities appeared on the 3rd day and then existed for a day, after that, the larvae having deformities on the 5^{th} day existed until day 10. On the other hand, at the concentration of 150 µg/L, deformities appeared on the first day but only lasted for a day and that was repeated until the 6^{th} day; larvae carrying deformities on the 7^{th} day could live up to the 10^{th} day.

Thus, the high concentration of lead increases linearly the death rate and deformation of the *Oryzias curvinotus* at the larval stage.

4. Conclusion

In this study, at the concentration of Pb^{2+} ion surveyed from 0-150 µg/L, the minimum concentration of Pb^{2+} ion affecting the survival rate of the larval *Oryzias curvinotus* was 50 µg/L and the viability of the larvae which was affected the most significantly was on the 5^{th} day at the concentration of 150 µg/L. The increased lead concentation would increased linearly the deformation of the *Oryzias curvinotus* at the larval stage from 1-10 days old. The results of this study showed that, through lead ion contaminated treatments, the higher the Pb^{2+} concentration was, the lower the survival rate was and the higher the possibility of spine deformation in the larvae was.

❖ ***Conflict of Interest:*** *Author have no conflict of interest to declare.*

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ẢNH HƯỞNG CỦA ION CHÌ (Pb^{2+}) LÊN TỈ LỆ SỐNG VÀ DỊ TẬT XƯƠNG SỐNG CỦA ẤU TRÙNG CÁ SÓC *Oryzias curvinotus* GIAI ĐOẠN 1-10 NGÀY TUỔI

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TÓM TẮT

Ion chì là một trong những cation có tác động lên sức sống, khả năng sinh trưởng, dị tật về xương sống trong quá trình sống của cá. Nghiên cứu được tiến hành nhằm đánh giá tác động của ion chì (Pb^{2+}) ở các nồng độ 0 µg/L; 50 µg/L; 100 µg/L; 150 µg/L lên quá trình phát triển cá Sóc giai đoạn ấu trùng sau nở từ 1-10 ngày tuổi. Kết quả cho thấy: (i) ở các nồng độ Pb^{2+} được kiểm tra khác nhau, nồng độ tối thiểu Pb^{2+} ảnh hưởng đến tỉ lệ sống của cá Sóc là 50 µg/L; (ii) ở mọi nồng độ được kiểm tra Pb^{2+}, tỉ lệ sống của cá bị ảnh hưởng nhiều nhất vào ngày thứ 5 tại nồng độ 150 µg/L. Dựa vào kết quả thu được, chúng tôi thiết lập một phương trình để dự đoán tỉ lệ sống của ấu trùng cá và tìm được nồng độ LD_{50} Pb^{2+} gây chết cá ấu trùng sau 10 ngày nuôi là 98 µg/L và LT_{50} của Pb^{2+} ở nồng độ 150 *µg/L* là 9,76 ngày. *Kết quả nghiên cứu cho thấy, nồng độ Pb^{2+} càng cao càng làm giảm tỉ lệ sống của cá, tăng dị tật xương sống cá ấu trùng và ảnh hưởng cao nhất tại nồng độ 150 µg/L.*

Từ khóa: ion chì; nhiễm độc kim loại nặng; cá ấu trùng; cá Sóc *Oryzias curvinotus*