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Growth and Immunity Performance of Nile Tilapia (*Oreochromis niloticus*) Challenged by Toxicity of Bio-Insecticide with Active Ingredients Eugenol and Azadirachtin

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ABSTRACT

This study aims to determine the maximum concentration and the long-term effects after exposure to a bio-insecticide with active ingredients eugenol and azadirachtin on the survival rate, immunity, and growth of Nile tilapia. The method used in this study was experimental, using a completely randomized design (CRD) with six treatments and three replications. Fishes were exposed to eugenol and azadirachtin at concentrations 10, 20, 30, 40, and 50% of LC_{50} value for 14 days, followed by 14 days of maintenance to see the effect on growth. The results showed that 66 $mg.L^{-1}$ treatment was a concentration that did not interfere with the survival rate of Nile tilapia, which was 86.7%. The number of leukocytes increased on the third day by the highest increase in 66 $mg.L^{-1}$ treatment at 12.01×10^4 $cells.mm^{-3}$. Meanwhile, erythrocytes decreased, with the highest decrease in 66 $mg.L^{-1}$ treatment at 1.13×10^6 $cells.mm^{-3}$. The average growth rate in fish slowed down with increasing concentrations of exposure, with the lowest average growth in length and absolute weight in the 66 $mg.L^{-1}$ treatment was 0.57 cm and 1.68 g.

INTRODUCTION

Pesticides are one of the most toxic contaminants entering the aquatic environment (Yang et al. 2021). Farmers usually use chemical pesticides to eradicate these pests because many are sold in the market and are very effective in eradicating pests (Astuti & Widyastuti 2016). Various aquatic organisms are physiologically affected by pesticides produced by the agricultural industry. For this reason, there is concern about releasing pesticides into aquatic environments worldwide. Chronic exposure to toxic substances in fish in the aquatic environment causes morphological, biochemical, and physiological cell changes (Fernandes et al. 2013). However, over time, bio-pesticides have become an alternative trend of choice for overcoming bio-pest problems because they can reduce environmental pollution and are relatively cheaper than chemical pesticides (Wiratno & Trisawa 2013). According to the Indonesian Ministry of Agriculture, the use of pesticides continues to increase yearly, with the most used being insecticides.

Among bio-insecticides, several plant families with the potential as a source of bio-insecticides are Meliaceae, Annonaceae, Piperaceae, Asteraceae, and Zingiberaceae (Prijono 1999). Neem seeds (*Azadirachta indica* A. Juss) and clove oil are known to be used as bio-insecticides to control insects (Deyashi et al. 2016). Neem seed extract and clove oil contain several active compounds that can act as contact poisons, such as azadirachtin and eugenol. Azadirachtin is a secondary metabolite of the triterpenoid group, which has long been used as an active ingredient in bio-insecticide from the neem plant (Deyashi et al. 2016). Meanwhile, 80% of the eugenol content was obtained from the extraction of the clove plant (*Eugenia caryophyllata* Thunb) in the form of clove oil or essential oil (Hadi 2013).

The extensive use of chemical insecticides has become essential to current agricultural practices (Deyashi et al. 2016). Using insecticides in the agricultural sector can produce waste and enter water bodies through agricultural irrigation. Jannah and Yusnita (2020) state that insecticides are persistent, and their residues can remain in the water, soil, and fish body tissues. Irrigation water is used for agricultural activities, usually also used for aquaculture activities. Nile tilapia cultivation can be done in semi-intensive ponds, intensive ponds, and even in rice fields. So, if the location of the aquaculture pond is located in an agricultural area, the

insecticide can enter and cause harmful effects on the life of aquatic organisms.

According to Pramleonita et al. (2018), Nile tilapia has a reasonably high tolerance for changes in its environment. In addition, Nile tilapia is also recommended by the USEPA (United States Environmental Protection Agency) as a toxicological test animal because it is widespread, easy to cultivate, can tolerate unfavorable environments, and is easy to maintain in the laboratory (Radiopoetra 1996). Water pollution due to insecticide use can be determined through toxicity tests. The use of experimental animals to conduct toxicity tests is a form of aquatic toxicology research whose role is to determine the level of toxicity of insecticide at specific concentrations.

The active compounds of bio-insecticide with active ingredients eugenol and azadirachtin are organic chemical compounds that can disrupt the environmental balance of fish in aquaculture ponds. In addition, the bio-insecticide has yet to be circulated in the community, and its toxicity to fish has yet to be discovered. Therefore, it is necessary to carry out further research to determine the long-term effect of post-exposure bio-insecticide with active ingredients of eugenol and azadirachtin on the survival rate, immunity, and growth of Nile tilapia.

MATERIALS AND METHODS

Chemicals

The bio-insecticide used has active ingredients of eugenol 20 $g.L^{-1}$ and azadirachtin 0.02 $g.L^{-1}$ that are in liquid form and have a volume of 500 mL/bottle. The bio-insecticide used has yet to be circulated among the public and is still in the testing phase. So, it doesn't have a trademark yet.

Animal Collection and Acclimatization

Freshwater teleost, *Oreochromis niloticus* (Nirwana Nile tilapia) fingerlings size 5-6 cm from the Cibiru Fish Fingerling Center, Bandung City, Indonesia. The experiments are performed following local/national guidelines for experimentation on animals. They were acclimatized in the hatchery for three days at 28 – 30°C. The fish are fed commercially available fish food with a 39 – 41% protein content.

Insecticide Preparation

Bio-insecticide was measured using a 200 µL micropipette. Each treatment, namely B treatments (13.2 $mg.L^{-1}$), C (26.4 $mg.L^{-1}$), D (39.6 $mg.L^{-1}$), E (52.8 $mg.L^{-1}$), and F (66 $mg.L^{-1}$), then put into a 1.5 mL Eppendorf tube and closed tightly. Then, affix a marker label.

Determination of LC_{50} Value

Fish were saved by twelve aquariums (six treatments and two replications). While the other ten fish were in the experimental group, the first group functioned as the control group. Fish from the experimental groups were exposed for 96 hours to eugenol and azadirachtin at various concentrations (50, 75, 100, 125, 150, and 175 $mg.L^{-1}$) prepared from the stock solution. Fish mortality in various concentrations was measured in 24, 48, 72, and 96 hours, and dead animals were removed immediately. Using the software, the LC_{50} values for 24, 48, 72, and 96 hours were calculated by converting mortalities (% values) into probit scale. Eugenol and azadirachtin were supplied at various safe concentrations (10, 20, 30, 40, and 50%) following the measurement of the LC_{50}.

Experimental Procedure

This research was conducted for 28 days by observations in the first 14 days and the next 14 days. This study used eighteen aquariums measuring 60 × 29.5 × 35.5 cm^3 as containers. Nile tilapia fingerlings used as many as 20 fish/aquarium with a volume of 40 L of water. The feeding rate was 3% of the weight of fish biomass by giving it three times a day at 08.00, 12.00, and 16.00 Western Indonesia Time. The commercial feed used contains 39-41% protein. To maintain water quality, siphoning off 10% of the maintenance container is carried out every once in three days in the afternoon. Meanwhile, on the 14 days, the water is changed.

The research method was experimental by completely randomized design (CRD) with six treatments and three replications. The concentrations used were 0%, 10%, 20%, 30%, 40%, and 50% of the LC_{50} of bio-insecticide for Nile tilapia. The following are the treatments used:

A Treatment: 0 $mg.L^{-1}$ (control)

B Treatment: 13.2 $mg.L^{-1}$ (10% of LC_{50})

C Treatment: 26.4 $mg.L^{-1}$ (20% of LC_{50})

D Treatment: 39.6 $mg.L^{-1}$ (30% of LC_{50})

E Treatment: 52.8 $mg.L^{-1}$ (40% of LC_{50})

F Treatment: 66 $mg.L^{-1}$ (50% of LC_{50})

Data Analysis

Data analysis on survival and growth rates used an analysis of variance (ANOVA) with an F test at a 95% confidence level. If a significant difference exists, a follow-up test with Duncan's multiple range test will be carried out at the 95% confidence level. Data analysis on leukocytes and erythrocytes was analyzed descriptively and quantitatively.

Macroscopic symptom data, feed response, and shock response were analyzed descriptively and qualitatively. Meanwhile, water quality data were analyzed descriptively and quantitatively and compared based on the Indonesian National Standard.

RESULTS

LC_{50} Values of Eugenol and Azadirachtin

The LC_{50} values of eugenol and azadirachtin in Nile tilapia are presented in Table 1; bio-insecticide of eugenol and

Table 1: The LC_{50} values of eugenol and azadirachtin in Nile tilapia following 96 h exposure

Confidence Limits							
	Probability	95% Confidence Limits for Concentration			95% Confidence Limits for log (Concentration)[b]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	59.233	.534	87.182	1.773	-.273	1.940
	.020	65.063	1.052	92.251	1.813	.022	1.965
	.030	69.057	1.616	95.712	1.839	.208	1.981
	.040	72.221	2.231	98.467	1.859	.349	1.993
	.050	74.902	2.899	100.820	1.874	.462	2.004
	.060	77.263	3.621	102.915	1.888	.559	2.012
	.070	79.393	4.398	104.831	1.900	.643	2.020
	.080	81.351	5.233	106.619	1.910	.719	2.028
	.090	83.173	6.127	108.312	1.920	.787	2.035
	.100	84.886	7.081	109.933	1.929	.850	2.041
	.150	92.363	12.831	117.502	1.965	1.108	2.070
	.200	98.773	20.391	125.036	1.995	1.309	2.097
	.250	104.625	29.978	133.476	2.020	1.477	2.125
	.300	110.176	41.660	143.969	2.042	1.620	2.158
	.350	115.582	55.117	158.339	2.063	1.741	2.200
	.400	120.956	69.368	179.589	2.083	1.841	2.254
	.450	126.394	82.890	212.067	2.102	1.919	2.326
	.500	131.985	94.493	261.065	2.121	1.975	2.417
	.550	137.822	103.987	332.920	2.139	2.017	2.522
	.600	144.018	111.847	436.754	2.158	2.049	2.640
	.650	150.715	118.657	587.664	2.178	2.074	2.769
	.700	158.110	124.903	812.338	2.199	2.097	2.910
	.750	166.498	130.977	1161.161	2.221	2.117	3.065
	.800	176.363	137.258	1738.962	2.246	2.138	3.240
	.850	188.602	144.227	2798.588	2.276	2.159	3.447
	.900	205.216	152.763	5117.303	2.312	2.184	3.709
	.910	209.443	154.813	5923.656	2.321	2.190	3.773
	.920	214.134	157.041	6945.614	2.331	2.196	3.842
	.930	219.414	159.494	8275.653	2.341	2.203	3.918
	.940	225.464	162.243	10066.596	2.353	2.210	4.003
	.950	232.569	165.394	12590.053	2.367	2.219	4.100
	.960	241.202	169.124	16379.101	2.382	2.228	4.214
	.970	252.256	173.760	22642.483	2.402	2.240	4.355
	.980	267.739	180.024	34841.845	2.428	2.255	4.542
	.990	294.093	190.180	68790.241	2.468	2.279	4.838

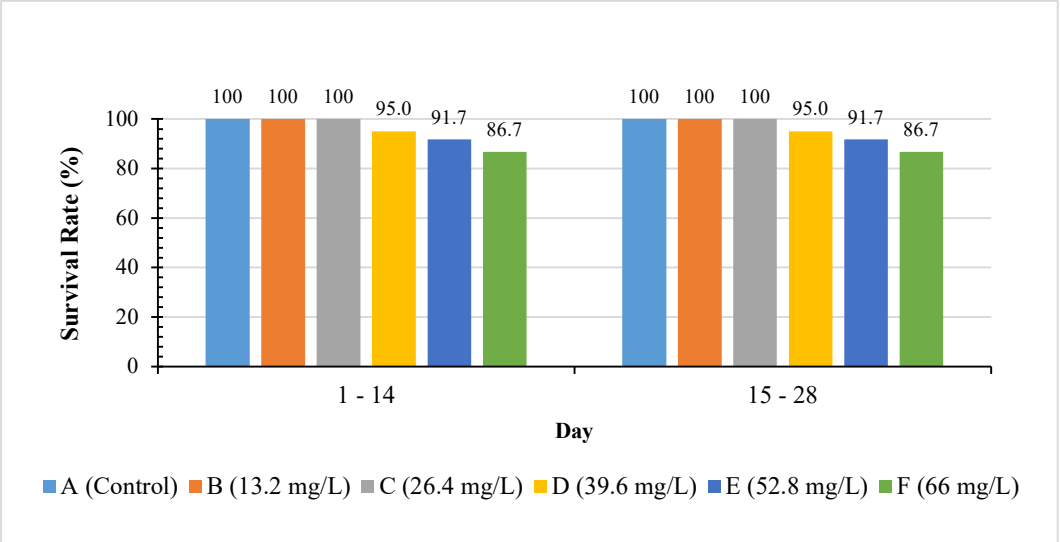

Fig. 1: The survival rate of Nile tilapia exposed to bio-insecticide with active ingredients eugenol and azadirachtin in the first 14 days and the second 14 days.

azadirachtin displayed relatively low toxicity to Nile tilapia, with an $LC_{50\text{-}96h}$ of 132 $mg.L^{-1}$.

Survival Rate (SR)

The survival rate of Nile tilapia was observed from the first day to the 14th day and continued from the 15th day to the 28th day, which can be seen in Fig. 1.

In the first 14 days of observation, the effects of eugenol and azadirachtin on survival rate in Nile tilapia was seen after 24 h exposure that there was death on 39.6, 52.8, and 66 $mg.L^{-1}$ treatments. The survival rate of Nile tilapia was significantly decreased at 66 $mg.L^{-1}$ treatment compared to the controls. Given that eugenol and azadirachtin survival rate changes in Nile tilapia, we hypothesized that the higher

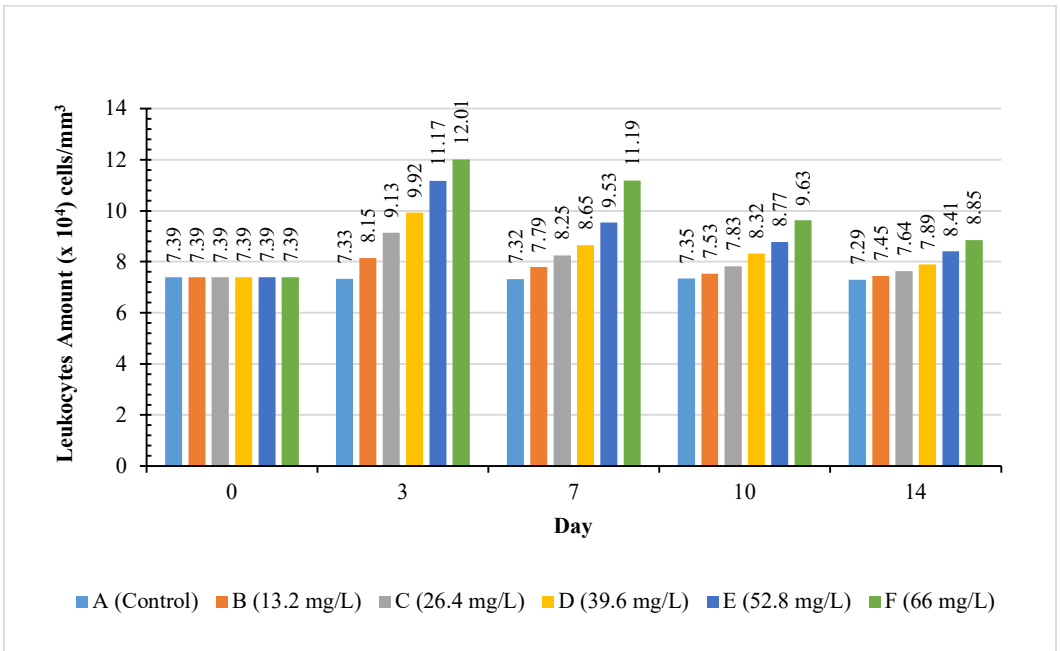

Fig. 2: The average number of Nile tilapia leukocytes in the first 14 days of observation with five times data collection.

the exposure concentration of bio-insecticide, the lower the survival rate of the tested fish. In the following 14 days of observation, there were no deaths in the test fish.

White Blood Cells (Leukocytes)

Based on Fig. 2, the leukocyte value in the treatment exposed to bio-insecticide experienced a maximum increase on the third day, with the highest increase in 66 mg.L^{-1} treatment at 12.01 × 10^4 cells.mm^{-3}. If compared to the control group, there was a significant decrease in this treatment. The increase in the number of leukocytes was higher than the control treatment can be used as a reference that fish health is being disturbed. On the seventh day, the leukocyte count of fish from each treatment decreased until the 14th day.

Red Blood Cells (Erythrocytes)

The number of erythrocytes in Nile tilapia exposed to bio-insecticide with the active ingredients eugenol and azadirachtin is still within the normal range for the number of erythrocytes in Nile tilapia but decreased compared to the control treatment. On the third day, it was seen that there was a maximum decrease in the number of erythrocytes in the treatment exposed to bio-insecticide, with the most significant decrease in 66 mg.L^{-1} treatment at 1.13 × 10^6 cells.mm^{-3}. On the seventh day, the number of erythrocytes in Nile tilapia exposed to bio-insecticide with the active ingredients eugenol and azadirachtin began to increase until the 14th day. The increase in erythrocytes on the seventh day is the initial stage of healing in fish by producing erythrocytes. The graph of the erythrocytes in Nile tilapia exposed to bio-insecticide with the active ingredients eugenol and azadirachtin can be seen in Fig. 3.

Length Growth (L_m)

Based on Fig. 4, the highest mean absolute length was in the control treatment at 1.88 cm. Conversely, 66 mg.L^{-1} treatment at 0.57 cm was the lowest length growth average. Length value absolute tended to decrease with increasing concentration of treatment. Exposure to bio-insecticide with active ingredients eugenol and azadirachtin significantly affected the length of growth of Nile tilapia fingerlings.

Weight Growth (W_m)

Based on Fig. 5, it is known that the highest average absolute weight growth was in the control treatment at 3.65 g. In the treatment exposed to bio-insecticide with the active ingredients eugenol and azadirachtin, the weight growth of Nile tilapia fingerling slowed down with the high concentration of the bio-insecticide given, with the lowest weight growth occurring in 66 mg.L^{-1} treatment at 1.68 g. The stunted growth of Nile tilapia can be seen in Fig. 6, the blackish digestive organs of the fish, presumably accumulated residues of bio-insecticide with the active ingredients eugenol and azadirachtin.

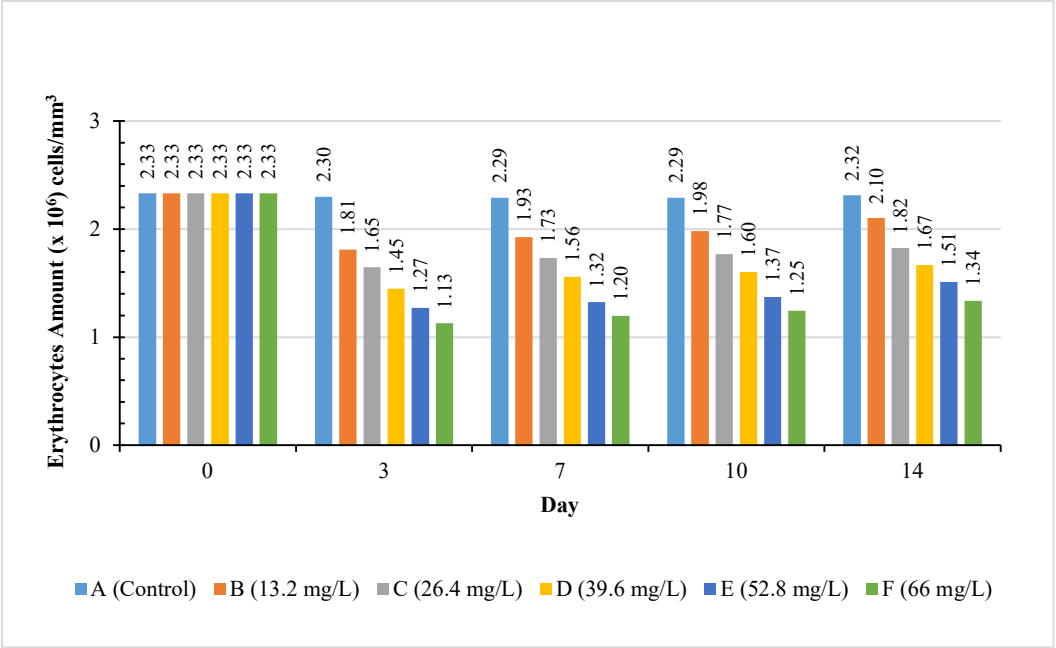

Fig. 3: The average number of Nile tilapia erythrocytes in the first 14 days of observation with five times data collection.

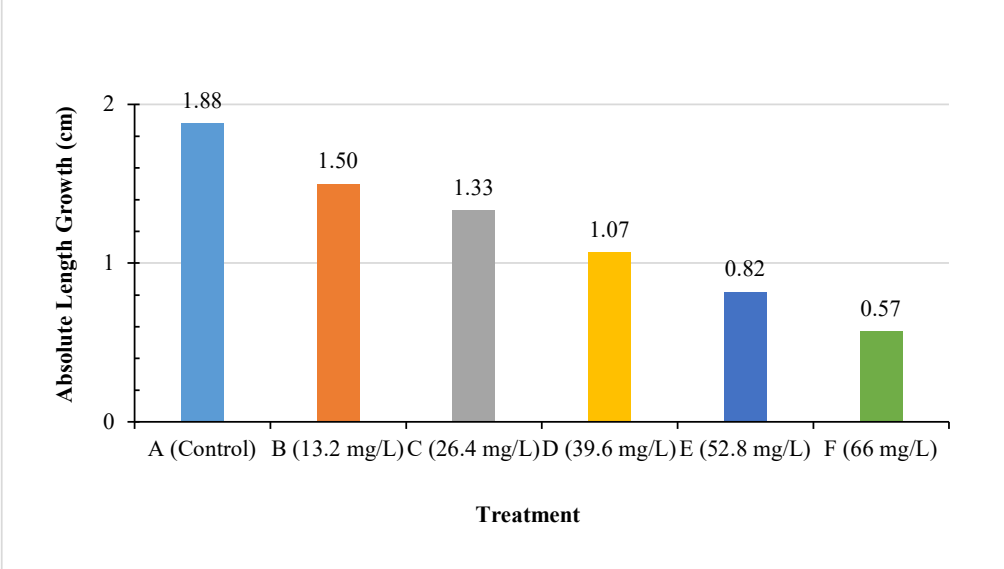

Fig. 4: The results of observing the growth in absolute length of Nile tilapia exposed to bio-insecticide with active ingredients eugenol and azadirachtin, which are carried out once a week.

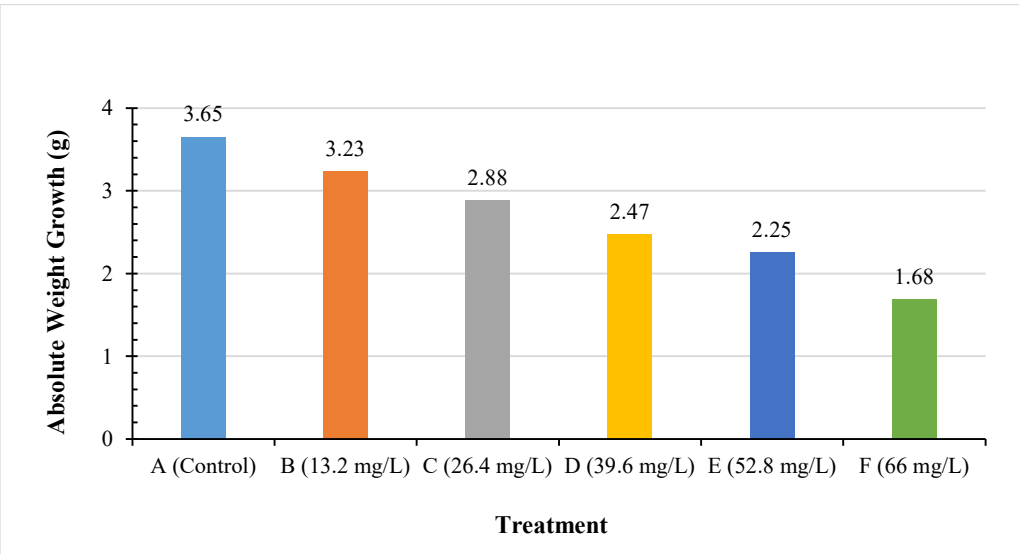

Fig. 5: The results of observing the growth in absolute weight of Nile tilapia exposed to bio-insecticide with active ingredients eugenol and azadirachtin, which are carried out once a week.

Macroscopic Symptoms

Observations of macroscopic symptoms were observed from the first until the 14th day. Based on Fig. 7, macroscopic symptoms on the morphology of the fish's body were shown on the first day, incredibly shortly after exposure to bio-insecticide with the active ingredients eugenol and azadirachtin, the color of the Nile tilapia's fins faded, and the body produced much mucus. In this 14th-day test, macroscopic symptoms in fish were not very visible (only showed mild symptoms).

Feed Response

Based on Table 2, the 39.6, 52.8, and 66 mg.L^{-1} treatments decrease appetite, especially on the first and second day, although not drastically. Then, gradually back to normal on the third day. Meanwhile, feed response in 13.2 and 26.4

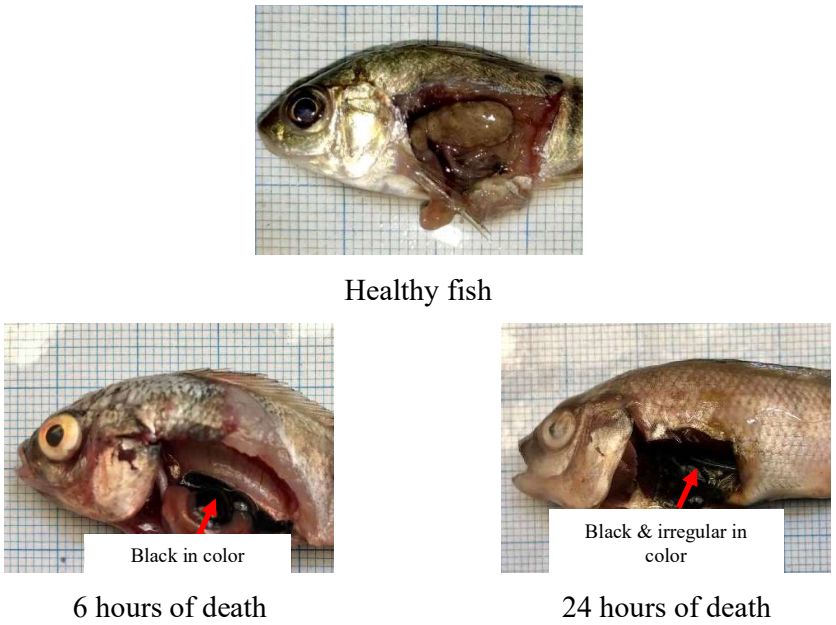

Fig. 6: Digestive conditions of Nile tilapia exposed to bio-insecticide with active ingredients eugenol and azadirachtin for the first 14 days.

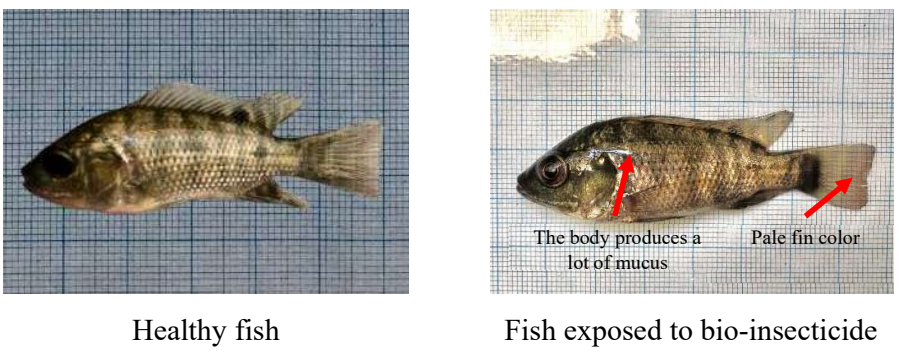

Fig. 7: Macroscopic symptoms of Nile tilapia visible during 14 days of rearing after exposure to bio-insecticide with active ingredients eugenol and azadirachtin.

mg.L^{-1} treatments showed that Nile tilapia feed response did not slow down.

Shock Response

Based on Table 3, the shock response of Nile tilapia in 52.8 and 66 mg.L^{-1} treatments on the first day shows that fish tend to be more passive and less responsive to shock. This is presumably due to exposure to toxicants, which causes the fish's metabolism to decrease, causing slower movements and less responsiveness to shocks. The activity of the fish gradually improved on the second day, and it was seen that in 52.8 mg.L^{-1} treatment in all replicates, > 80% of the fish responded to the shock given. In the control treatment, 13.2, 26.4, and 39.6 mg.L^{-1} treatments, the fish's shock response tended to be stable and active from the first day of exposure to the 14th day.

Water Quality

During the first 14 days of rearing, the water temperature was 28-32°C. The temperature range for the first 14 days of maintenance is known to be not much different from the maintenance for the next 14 days. The range of pH values of all treatments during the first 14 days of maintenance was in the range of 6.5-6.8. Meanwhile, the maintenance for the next 14 days was in the range of 6.6-6.7. The dissolved oxygen measurements showed that the lowest dissolved oxygen content was 4.8 mg.L^{-1}. Meanwhile, the highest dissolved oxygen value was 5.6 mg.L^{-1}. The results of water

Table 2: Response of Nile tilapia feed exposed to bio-insecticide with active ingredients eugenol and azadirachtin from the first day to the 14th day.

Treatment	Repeat	Day													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
D	1	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
E	1	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
F	1	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Description: (+++) fish respond to feed in under 30 seconds, (++) fish respond to feed in 31 - 90 seconds, (+) fish respond to feed in 91 - 180 seconds, (-) fish do not eat the feed given

Table 3: Response of Nile tilapia shock exposed to bio-insecticide with active ingredients eugenol and azadirachtin from the first day to the 14th day.

Treatment	Repeat	Day													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
D	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
E	1	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
F	1	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Description: (+++) > 80% of fish respond to shock, (++) > 60% of fish respond to shock, (+) 30 - 60% of fish respond to shock, (-) < 30% of fish respond to shock.

Table 4: Water quality (temperature (°C), dissolved oxygen ($mg.L^{-1}$), and (pH)).

Treatment	3rd, 7th, 10th, and 14th day			21st and 28th day		
	Temperature [°C]	pH	DO [$mg.L^{-1}$]	Temperature [°C]	pH	DO [$mg.L^{-1}$]
A	30 – 32	6,5 – 6,7	5,1 – 5,6	30 – 32	6,6 – 6,7	5,3 – 5,5
B	28 – 30,5	6,5 – 6,8	4,8 – 5,6	29 – 31	6,6 – 6,7	5,4 – 5,6
C	28,5 – 31	6,5 – 6,7	4,9 – 5,4	29,5 – 31	6,6 – 6,6	5,2 – 5,4
D	28,5 – 31	6,6 – 6,7	5,1 – 5,5	29 – 31	6,7 – 6,7	5,3 – 5,5
E	28 – 31	6,5 – 6,7	5,0 – 5,4	28,5 – 30,5	6,6 – 6,6	5,3 – 5,4
F	28,5 – 31	6,6 – 6,7	5,0 – 5,4	28,5 – 31	6,7 – 6,7	5,2 – 5,4
Optimal (INS 2009)	25 - 30	6,5 - 8,5	≥ 5	25 - 30	6,5 - 8,5	≥ 5

quality measurements (temperature, dissolved oxygen (DO), and potential of hydrogen (pH)) are still within the optimal range because it is so controllable according to the demands of a completely randomized design (CRD); there should be no other factors other than exposure to bio-insecticide with active ingredients eugenol and azadirachtin. Water quality during research can be seen in Table 4.

DISCUSSION

Based on this study, bio-insecticides with active ingredients eugenol and azadirachtin are compounds with a low toxicity level to Nile tilapia fingerlings because the $LC_{50\text{-}96h}$ value shows > 100 $mg.L^{-1}$, which is 132 $mg.L^{-1}$. These results follow the criteria determined by the USEPA that the $LC_{50\text{-}96h}$ toxicity value of > 100 $mg.L^{-1}$ in the aquatic environment is classified as a low toxicity category (Kinasih et al. 2013). It is also supported by the results of the first 14 days of observation that the lowest survival rate value at 66 $mg.L^{-1}$ exposure (50% LC_{50}) was 86.7% which showed that it was still relatively good for the survival rate of Nile tilapia but caused the most deaths with the same symptoms as in exposure 39.6 and 52.8 $mg.L^{-1}$. This follows the quality standards of the Indonesian national average that the survival rate value of good Nile tilapia has a percentage of ≥ 75%. In contrast, at exposures of 13.2 and 26.4 $mg.L^{-1}$, there were no deaths, and the clinical symptoms were not significant. This shows that the higher the concentration of bio-insecticide given, the lower the survival rate of the test fish. It is known that on the following 14 days of observation, there were no deaths in Nile tilapia after exposure to bio-insecticides, which was allegedly due to water changes. Hence, the fish's living media supported everyday life. In addition, Nile tilapia is a fish that easily adapts to its environment. This is supported by the statement of Sibagariang et al. (2020) that Nile tilapia is a freshwater fish that easily adapts to unfavorable environments.

Environmental pollution due to the extensive use of pesticides without proper management has a broad impact on the potential survival rate of aquatic animals, especially fish (Mishra & Devi 2014). This situation is caused by the function of the gills and organs directly related to the fish's living environmental media starting to experience damage due to insecticide contamination. Fish are exposed to chemical contaminants due to direct integumentary contact through the mouth and respiration through the gills (Shah & Parveen 2020). Some of these toxic chemicals persist in the environment for a long time and can affect physiology (cause damage to tissues) (Mishra & Devi 2014). The gills cannot supply oxygen adequately to the body (Ihsan et al. 2021). However, in lower concentrations, this toxic complex can cause increased tissue damage and reduce fish survival rate (Yancheva et al. 2022). In summary, it can be concluded that in a short-term trial by Georgieva et al. (2021), gills are more severely affected as the principal organs that come into direct contact with poisons when pollution occurs in the aquatic environment. As explained by Fernandes (2019), gills are a crucial entry point for dissolved pollutants due to their extensive surface area, and morphological characteristics support the absorption of contaminants in fish.

According to the research results of Georgieva et al. (2021), the significant impact of the pesticides used can have a long-lasting effect on the liver through the bloodstream. The fish's respiration rate reflects metabolic activity and responses due to changes in the surrounding environment, which can indicate adjustment capacity (Yancheva et al. 2022). Fish can reflect a direct response to toxic substances contained in water by changing their physiological response, such as the intensity of respiration rate (Yancheva et al. 2022). Similar to the results obtained, Nile tilapia exposed to bio-insecticide with active ingredients eugenol and azadirachtin experienced a lack of oxygen supply compared to the control group. These results can generally be attributed to respiratory distress caused by the limited access to oxygen to the fish's gills. This condition was also one of the factors causing the decrease in the number of erythrocytes compared to the control after exposure to bio-insecticide with the active ingredients eugenol and azadirachtin. Ikeda

(1970) stated that a decrease in metabolic rate decreased the number of fish erythrocytes. A decrease in the number of erythrocytes indicates the occurrence of kidney disorders and fish suffering from anemia (Wedemeyer & Yasutake 1977). Possibly, anemia occurs due to reduced oxygen binding in the blood due to disruption of the fish's respiratory organs, so the release of erythrocytes in the blood circulation is minor. According to Matofani et al. (2013), hemoglobin is closely related to erythrocytes; the lower the hemoglobin level, the fish is suspected of having anemia because it is closely related to the oxygen-holding capacity in the blood.

The clinical symptoms shown by the fish were when they were exposed to bio-insecticide with the active ingredients eugenol and azadirachtin, in which the fish soared to the surface, hanging from the surface to take in oxygen, and the fish's body produced much mucus. This study's results align with Yancheva et al. (2022) that excessive mucus secretion may result from a defensive response and avoidance to minimize irritation caused by the tested pesticides. According to Uchenna et al. (2022), mucosal cells can efficiently capture toxic substances and help prevent the entry of harmful substances into the gills so that the release of mucus can reduce the entry of various toxic compounds into the circulatory system. Epidermal mucus functions as a physiological and immunological first line of defense to maintain normal physiological status in teleost fish (Santoso et al. 2020). However, mucus in the fish's body can be a problem if much mucus is produced because it can inhibit gas exchange through the gills (Ihsan et al. 2021). Exposure to toxic substances can cause stress to fish and alter mucus production, harming fish (Reverter et al. 2018). In line with the statement of Santoso et al. (2020), fish that are stressed due to exposure to chemical pollutants secrete more mucus as a barrier and inhibit the diffusion of chemicals.

Based on visual observations immediately after exposure to bio-insecticides with the active ingredients eugenol and azadirachtin, there were changes in the behavior of Nile tilapia, such as irregular swimming patterns, whirling, and tending to tilt. According to the research results of Ihsan et al. (2021), fish exposed to chlorpyrifos inhibit metabolic processes in the body, causing fish to experience stress. The percentage of stressed goldfish with inactive and solitary fish tended to decrease with the time the insecticide was exposed. After being exposed to the insecticide chlorpyrifos, the common symptoms exhibited by goldfish are unusual swimming movements with irregular movements or classified as panic by moving in all directions. The next day, there was death in the fish, and the living conditions were more passive, silent, and solitary on the bottom or on the surface and getting weaker. So, it can be seen that the longer the fish are exposed to insecticides, the lower the movement of the fish's body (Ihsan et al. 2021). Fish infected with the insecticide chlorpyrifos generally experience a decrease in movement, which is thought to be because the chlorpyrifos has affected the nervous system and interfered with muscle movement, causing the fish to weaken and move erratically (Ihsan et al. 2021). Strange or abnormal fish movements are caused by a lack of coordination between the nervous system and muscles (Ihsan et al. 2019). According to Sellamuthu (2014), eugenol is also a phenol compound that has an alcohol group, so it can weaken and disrupt the nervous system. Based on this, it is known that the active compound of eugenol has a strong enough effect on weakening the nervous system in exposed Nile tilapia so that the fish give an abnormal response to their physiological system. Meanwhile, the cause of fish showing a whirling swimming pattern is caused by damage to the midbrain (metencephalon), which is responsible for regulating the body's balance in water (Hardi et al. 2011). The brain plays a regulatory role in fish physiology and is the most critical organ in fish toxicology, especially when pesticides are involved in the nervous system's workings (Mishra & Devi 2014).

Stress on fish due to exposure to contaminants, besides affecting the clinical symptoms, can also affect the number of leukocytes. This is supported by Lestari et al. (2017) that high leukocyte counts are due to stress on fish due to poor and polluted water quality. Based on the research journal Singh & Srivastava (2010), leukocytes play a significant role in the body's defense mechanism, which consists of lymphocytes (producing antibodies), granulocytes, and monocytes (as phagocytes to save injured tissue). The innate immune response is a frontline defense and a robust response, but it can be altered by pesticides (Li et al. 2013). The increase in the number of leukocytes higher than the control treatment can be used to reference that fish health is being disturbed due to exposure to bio-insecticides with active ingredients eugenol and azadirachtin. The increase in leukocytes in fish exposed to insecticides provides essential information about the general physiology and health status of fish under investigation (Singh & Srivastava 2010). It is known that fish exposed to triazophos 5 and 10% $LC_{50\text{-}96h}$ show significant reductions in various immune functions (Chandra et al. 2021).

Disruption of the homeostatic and physiological mechanisms of the fish body can cause lasting effects on inhibited growth and productivity of fish, which is characterized by fish's appetite loss (Royan et al. 2014). The decrease in appetite and the startle response in fish is thought to be due to the effects of exposure to the active ingredients eugenol and azadirachtin, which cause the fish's

metabolism to decrease so that their movements slow down and are less responsive when they are shocked. This is supported by the statement of Wardhana and Wijaya (2015) that essential oils have the potential to inhibit eating activity. Meanwhile, the feed response in treatments 13.2 and 26.4 $mg.L^{-1}$ showed that the Nile tilapia feed response did not slow. This shows that bio-insecticide with active ingredients eugenol and azadirachtin at exposure to these concentrations does not affect the fish's sense of smell and sight, so the fish can respond to feed in under 30 seconds. This statement is supported by the results of Suryadi et al. (2021), which stated that a fungicide with the active ingredient *Bacillus amyloliquefaciens* did not interfere with the sense of smell and sight of Nile tilapia and carp in responding to feed.

This research showed that the growth in length and absolute weight of Nile tilapia exposed to bio-insecticide with active ingredients eugenol and azadirachtin slowed down as the bio-insecticide exposure increased. The inhibition of fish growth is thought to be due to reduced oxygen supply due to disruption of the respiratory organs. Aquatic biota need oxygen in burning food to carry out activities, such as swimming, growth, reproduction, etc. (Ihsan et al. 2021). Therefore, a lack of oxygen in the fish's body can disrupt fish life, including slowing growth (et al. 2019). Similar to the results of Hafiz et al. (2018) on the toxicity of the herbicide isopropylamine glyphosate on the growth of catfish fingerlings, which showed that the highest growth was in the control treatment of 5.76 ± 1.40^{b} and the lowest in the 30% LC_{50} treatment (2.90×10^{-1} $mL.L^{-1}$) of 3.73 ± 1.51^{a}. The effect of pesticides on fish can occur indirectly (sublethal effects), which can inhibit growth (Hafiz et al. 2018). This was also stated by Damayanti & Abdulgani (2013) that behavioral abnormalities in fish, when exposed to organophosphates, can fail to store energy for metabolic processes.

The stunted growth of Nile tilapia can be seen in Fig. 6, the blackish digestive organs of the fish, presumably due to the accumulation of bio-insecticide residues with the active ingredients eugenol and azadirachtin. Thus, both directly and indirectly, the work function of the digestive organs of fish is disrupted and affects its growth. Exposure to toxic pesticides causes energy obtained from feed to be focused more on the adaptation and maintenance of damaged body tissue than on its growth (Mason 1979). Thus, the higher the concentration of exposure to bio-insecticide with the active ingredients eugenol and azadirachtin, it is suspected this will cause an imbalance between the amount of feed that enters as energy and energy output for survival rate. This is what causes the feed to be less effective for the growth of Nile tilapia.

CONCLUSIONS

The recommended concentration of bio-insecticide with active ingredients eugenol and azadirachtin is 66 $mg.L^{-1}$ (50% LC_{50}), which is a concentration that does not interfere with the survival rate of Nile tilapia. In addition, it is necessary to conduct field tests to compare the doses sprayed on plants with a concentration of bio-insecticide getting into the waters.

The number of leukocytes increased on the third day, with the highest increase in 66 $mg.L^{-1}$ treatment at 12.01×10^{4} $cells.mm^{-3}$. Meanwhile, the number of erythrocytes decreased, with the largest decrease in 66 $mg.L^{-1}$ treatment at 1.13×10^{6} $cells.mm^{-3}$.

Fish feed response decreased on the first and second days, then gradually increased on the 3rd day. Meanwhile, the fish startle response decreased on the first day and gradually became active again on the third day.

The highest absolute length growth was in the control treatment at 1.88 cm, and the lowest absolute length growth was in the 66 $mg.L^{-1}$ treatment at 0.57 cm. Meanwhile, the highest absolute weight growth was in the control treatment at 3.65 g, and the lowest absolute weight was in the 66 $mg.L^{-1}$ treatment at 1.68 g.

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