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THE USE SIMPLE BIOMARKERS ON *Oryzias celebensis* EMBRYOS FOR TOXICITY DETERMINATION IN TALLO RIVER SEDIMENT SEMI-IN SITU

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Abstract: This study aimed to analyse the embryogenesis phase and contaminant-sensitive biomarkers in Tallo River sediments. Using the purposive sampling method, sediment sampling was conducted at three stations with three replicates each. Exposure of *Oryzias celebensis* embryos was performed using the quasi-experimental method. The exposure media used were control media, namely Embryo Rearing Medium (ERM), 0.25 mg/L Lead (Pb) Positive Control (PC) media, and Sediment Supernatant (SS) media from sediment sampling station (SS 1, SS 2, and SS 3). Biomarkers in this study include egg diameter, yolk volume, yolk absorption rate, somites, heart rate, jaw movement, hatching time, survival, and length of early hatched larvae. The result revealed that the highest concentration of Pb metal in the sediment was at station 2 and the lowest at station 3. Embryos exposed to SS 1 and SS 2 media predominantly demonstrated a significant comparison to embryos on ERM media. The crucial biomarkers for identifying contaminants in polluted waters include the survival rate of embryos, somites, heart rate, and yolk absorption rate. These biomarkers offer a more direct representation of the toxic impact of contaminants on essential biological processes, making them valuable indicators. Accordingly, biomarkers can be used for biomonitoring based on the effects of pollutants in sediments, waters, or biota.

Keywords: Biomarkers, embryo, *Oryzias celebensis*, sediment, Tallo River.

Introduction

The Medaka fish, belonging to the genus *Oryzias* are extensively utilised as highly reliable test organisms in various sectors, including ecotoxicology (Hilgers & Schwarzer, 2019; Audira *et al*., 2021). Notably, Medaka fish, specifically in their Early Life Stages (ELS) are susceptible to contaminants and play a crucial role in the ecosystem. As a result, they are often used as an early warning sign of ecosystem stress (Barhoumi *et al*., 2016). Burden *et al.* (2020) stated that using fish embryos represents an excellent opportunity to replace, reduce, and improve toxicity testing opportunities as performed on adult fish. As such, Medaka fish embryos have many advantages as test animals in detecting contaminants (Powe *et al*., 2018; Song *et al*., 2021).

Environmental pollution monitoring can be conducted through two principal methodologies: The classical approach and the modern approach, which employs biomarkers. The classical approach is centred on directly measuring the concentration of contaminants in the environment (Yaqin *et al*., 2019; 2022). However, this method does not always provide a comprehensive overview of the direct biological impact of contamination on organisms. Hence, with the advancement of science and technology, scientists have begun to employ biomarkers in ecotoxicology as tools or endpoints utilised in laboratory tests and also to conduct biomonitoring in the natural environment (Yaqin *et al*., 2019; Schuijt *et al*., 2021). Nonetheless, using biomarkers can provide researchers with more immediate information, which is often more useful in rapid field applications.

Techniques for detecting pollution of substances or chemicals in waters have now been developed using simple biomarkers. Biomarkers are biological responses of the

biological organisation of an organism to environmental stress (Yaqin *et al*., 2022). The concept of biomarkers is the preferred candidate as a monitoring tool to detect and assess the biological impact of pollution on organisms and environmental quality simultaneously (Yin *et al*., 2017). Researchers in the field of ecotoxicology have studied a variety of biomarkers. This includes those in *Oryzias latipes* (Jeon *et al*., 2016; Pannetier *et al*., 2019) and *Oryzias javanicus* (Kim *et al*., 2014; Nam *et al*., 2020) have been identified as possessing Acetylcholinesterase (AchE), Carboxylesterase (CE), Cytochrome P450 (CYP), and Glutathione S-Transferase (GST) enzymes. Yaqin (2019) posited that biomarkers could be quantified at the molecular or behavioural levels. However, some of these biomarkers necessitate the utilisation of sophisticated analytical tools. The employment of simple biomarkers can facilitate the detection of contaminants *in vivo* and *in situ* (Yaqin *et al*., 2020).

The biomarkers introduced in this study represent an advancement of traditional methods, particularly through the utilisation of *O. celebensis* embryos and a combination of simple, highly responsive biomarkers. The utilisation of biomarkers in embryos was selected since the embryonic phase is the most susceptible or responsive phase (Chen *et al*., 2020). Thus, it is anticipated to promptly exhibit a biological reaction to environmental stress. Pollutants, such as toxic metals, possess a property that allows them to rapidly bind and settle at the bottom of the water. Consequently, they accumulate in the sediment, producing higher metal concentrations than water (Saiki *et al*., 2021).

Sediments serve as long-term repositories for pollutants and as secondary reservoirs of contamination for aquatic animals. Sediments are widely recognised as significant repositories for environmental contaminants such as metals (Li *et al*., 2020; Saiki *et al*., 2021; Lordache *et al*., 2022). Furthermore, sediments are particularly relevant in ecotoxicology since they can provide realistic scenarios or depictions of environmental pollution (Schiwy *et al*., 2020). Compared to the analysis of water and biota, the chemical content of sediments can provide a more in-depth picture of water pollution (Kang *et al*., 2019). In addition, sediments contain more metals than water or organisms in the water (Barhoumi *et al*., 2016; Patang, 2018; Chen *et al*., 2020; Lordache *et al*., 2022). Since metals tend to settle and accumulate in sediments, they are often an indicator of metal pollution in aquatic environments. Note that the test animals used in this study were *O. celebensis* embryos.

O. celebensis is one of the species of Medaka fish in South Sulawesi (Mandagi *et al*., 2018). Thus far, no research has assessed the effects of pollutants using *O. celebensis* embryos, native to Sulawesi, with a semi-in situ approach to sediments in the Tallo River. The Tallo River is one of the most vital rivers for the people of Makassar. However, the river is under environmental pressure along its course. According to Rukminasari and Sahabuddin (2012), around the banks of the Tallo River, various activities and several industries are suspected of polluting by discharging their waste along the watershed of the Tallo River without prior treatment. Therefore, this study aims to analyse the effects of pollutants accumulated in Tallo River sediments using *O. celebensis* embryos semi-in situ using biomarkers to benefit effects-based biomonitoring. Accordingly, the biomarkers that will be used are simple biomarkers to determine the most sensitive ones in detecting pollutants in Tallo River sediments.

Materials and Methods

Sampling Collection

Sediment sampling was conducted at three stations using the purposive sampling method in Tallo River Waters, Manggala District, Makassar, South Sulawesi, Indonesia. The determination of the three stations was based on polluted waters. Station 1 is located in the Biring Romang tributary, which is a polluted area due to waste disposal from one of the industries around the Tallo River. Meanwhile, station

2 is located at Lakkang Pier, which is an area polluted by residential waste. Moreover, station 3 is located in the Bontoa tributary, which is a water flow area that empties into the sea; hence, it is suspected that there is an accumulation of pollutants in this area. The sampling locations can be observed in Figure 1.

Sediments were sampled from 0 to 10 cm from the sediment surface at the selected station. Consequently, sampling was performed using the Birge-Ekmen tool three times at each station. The sediment samples were transported to the laboratory and desiccated for at least 16 hours. After drying, the sediments were crushed into a fine powder and filtered through a 2 mm screen for subsequent treatment. Measurement of water quality parameters as supporting data was conducted at each sediment sampling station. The parameters measured were temperature (°C), Dissolved Oxygen (DO, mg/l), and pH. Water quality parameters were measured using the Water Quality Checker (WQC) tool. Correspondingly, measurements were conducted as many as three times in each station during sampling.

The Media Preparation

The media utilised for exposure in this investigation included control media (Embryo Rearing Medium (ERM), Positive Control (PC) media containing 0.25 mg/L of Lead (Pb) metal (PC), and Sediment Supernatant (SS) from each station (SS 1, SS 2, and SS 3). The components utilised in the production of ERM consisted of 10.0 g of Sodium Chloride (NaCl), 0.3 g of Potassium Chloride (KCl), 0.4 g of Calcium Chloride Dihydrate ($CaCl_2$ H_2O), 1.63 g of Magnesium Sulfate ($MgSO_4$), and 1 ml of Sodium Bicarbonate ($NaHCO_3$) solution, which was prepared by dissolving 0.25 g of $NaHCO_3$ in 20 ml of Water (H_2O). The chemicals were purchased from Merck, Germany. A pure Pb standard solution of 1000 mg/L was diluted to a final concentration of 0.25 mg/L. Note that dilution was performed using an ERM solution. The preparation of SS took reference from (Barhoumi *et al*., 2016), which has been modified. A total of 10 g of sediment has been mashed and mixed with 50 ml of ERM solution. Once the sediment and ERM were combined, they were vigorously agitated and thoroughly mixed for 15 minutes. Subsequently,

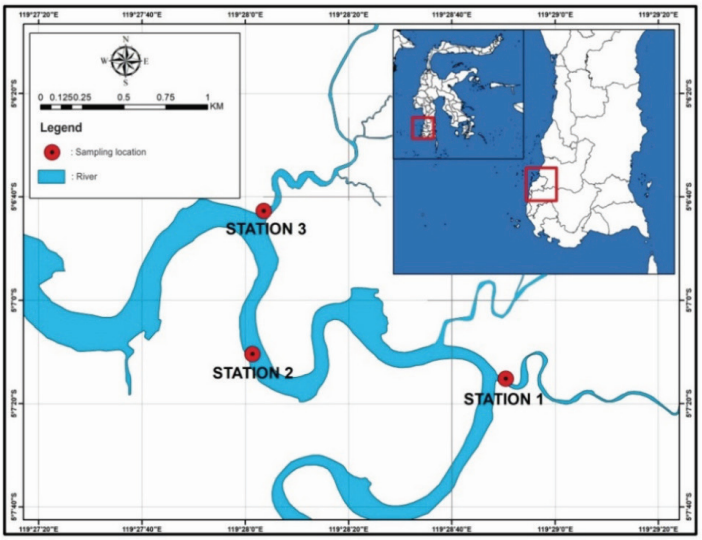


Figure 1: Geographic location of the study area in Tallo River Waters, Manggala District, Makassar, South Sulawesi, Indonesia, with the sediment sampling locations

the solution underwent centrifugation for 10 minutes at a temperature of 15°C. The liquid portion that remained after the solid settled was transferred into a container for analysis. The supernatant was intentionally not filtered to maintain its contamination concentration. Consequently, the liquid portion was preserved at a temperature of 4°C before its utilisation in subsequent examinations.

Analysis of Pb Metal in Sediment

A total of 10 g of fine sediment samples at each station were placed in plastic samples. The samples were then submitted to the Bogor Agricultural Institute testing, calibration, and certification services laboratory. The method used was Standard Methods for the Examination of Water and Wastewater APHA 23rd Edition (2017) Methods 3112; 3111B.

Fish Breeding and Embryo Collection

O. celebensis broodstock was kept in an aerated aquarium, with a male-to-female ratio of 2:1. The parents are fed otohime or Artemia pellets three times a day to support egg production. Note that spawning and fertilisation occur naturally in the aquarium. An aquarium with dimensions of 70 cm x 40 cm x 40 cm was utilised to accommodate the fish. During the rearing period of *O. celebensis* broodstock in the laboratory, the water temperature within the aquarium was maintained within a range of 26°C to 29°C and the pH was kept between 7 to 8. After the female lays eggs, the eggs are transferred to a petri dish containing ERM solution. The grapefruit-like eggs are separated from the filaments by gently twisting with the index finger. Fertilised eggs, characterised by perivitelin spaces are selected using a microscope.

Embryo Exposure

The study was conducted using a quasi-experimental methodology, incorporating five treatments (rearing media), each with 10 repetitions. The exposure medium was transferred into a 24-well microplate using a drop pipette. Here, 2 ml of rearing media was added to each hole of the microplate. Subsequently, each microplate was inserted with one fertilised egg (which has passed the egg selection stage). Embryos are exposed to contaminants starting from phase 17 (1 day-post fertilisation, dpf) until hatching. The embryo is exposed to the exposure media during its embryonic development period until it hatches into a larva. Embryos hatch into larvae usually around 9 to 11 dpf under control conditions. Note that embryos that die or do not hatch are removed from the microplate. During exposure, the embryos are maintained at 27°C.

Biomarker

The egg diameter will be measured by drawing a line vertically and horizontally. Egg diameter is obtained using the following formula (Rodriguez *et al*., 1995):

$$Ds = \sqrt{(Dh \times Dv)} \quad (1)$$

where Ds: Actual egg diameter (mm), Dh: Horizontal egg diameter (mm), and Dv: Egg diameter.

Measurement of yolk volume is used to evaluate the growth and metabolic rate of the embryo. Yolk volume can be calculated using the following equation (Wang *et al*., 2020):

$$YV = \frac{\pi}{6} \times LH^2 \quad (2)$$

where VK: Yolk volume (mm^3), L: Yolk length (mm), H: Yolk height (mm), and π: 3.1416. The yolk absorption rate will be calculated using the following formula (Heming & Buddington, 1988):

$$YAR = \frac{(Vo - Vt)}{T} \quad (3)$$

where YAR: Yolk absorption rate (mm^3/hour), V_o: Initial volume of yolk (mm^3), V_t: Final volume of yolk (mm^3), and T: Time (hour). The somite count was determined by observing and analysing images captured through a microscope. Note that the somite count commenced at stadia 19 to 21 (González-Doncel *et al*., 2005). The heart rate of *O. celebensis* fish embryos was manually measured using a camera and microscope, relying on digital data. The heart rate was assessed by measuring

the duration required to achieve 30 beats. The duration needed for fish embryos to achieve 30 beats was converted to the heart rate in beats per minute using the relevant calculation (Chen *et al*., 2020):

$$N = 30/T \times 60 \quad (4)$$

where N: Number of embryonic heartbeats per minute (beats/minute) and T: Time taken to reach 30 seconds. Jaw movement in *O. celebensis* embryos was observed under a microscope starting at time >194 dpf. Jaw movement is characterised by the lower jaw moving actively and allowing it to remain open (Le Bihanic *et al*., 2020). When the egg's chorionic membrane breaks and the larvae begin to move, it is time to observe the egg hatching. The hatched eggs will be monitored daily from the first day of hatching until every embryo in each rearing medium hatches. The term "hatch" refers to the complete departure of an embryo from the chorion; non-complete exits are considered non-hatching. Embryo survival can be calculated using the following equation (Tian *et al*., 2018):

$$SRe = \frac{N_t}{N_o} x\ 100\% \quad (5)$$

where SRe: Survival rate embryo, N_t: Number of embryos alive after hatching, and N_o: Number of fertilised embryos before hatching. Using the Image Raster 3.0 program, the total body length of every freshly hatched larva was measured. The larvae's total body length was measured from the point of the lower jaw to the tip of the caudal fin (Kataba *et al*., 2022).

Data Analysis

Data analysis uses statistical and descriptive analysis. Statistical analysis was conducted with GraphPad Prism 8 software using the Kurskal Wallis test to analyse the comparison of the number of somites, egg diameter, yolk volume, yolk absorption rate, heart rate, and heart size as well as the length of larvae at hatching and hatching time. Meanwhile, embryo survival data was analysed using survival analysis on GraphPad Prism 8 software. Moreover, descriptive analysis was performed by observing each phase of embryo development. The parameters observed were signs of damage to the embryo.

Results and Discussion

Concentration of Pb Metal in Sediment

Pb metal in sediment exhibited variations in concentration with the lowest value at station 3, i.e., 5.65 ± 1.24 mg/kg (range value 4.7 to 7.05 mg/kg), station 1, i.e., 12.79 ± 1.51 mg/kg (range value 11.8 to 14.5 mg/kg), and the highest at station 2, i.e., 34.38 ± 0.56 mg/kg (range value 33.75 to 34.75 mg/kg) (Figure 2). The high concentration of Pb metal at station 2, located at Lakkang Pier is thought to be polluted by residential waste and also as a stopping place for fishing boats in the surrounding community. According to Xiao *et al.* (2021), boat fuel is one of the primary sources of Pb metal that builds up in sediments. Boat emissions have the potential to disperse Pb into the surrounding environment, particularly when leaded gasoline is used. In addition, water environmental factors such as pH and DO affect metal interactions in sediments (Li *et al*., 2020; Miranda *et al*., 2021; Wang *et al*., 2023). The pH values at the three stations ranged from 6.72 to 6.86 and the average temperature was around 30°C. Meanwhile, DO values varied, with station 3 (5,02 ± 0,77 mg/l) > station 2 (3,09 ± 0,85 mg/l) > station 1 (1,74 ± 2,49 mg/l) (range value 1.74 to 5.02 mg/l).

The low DO at stations 1 and 2 is believed to cause high Pb concentrations and some industries around the Tallo River are suspected of polluting by dumping their waste along the watershed of the Tallo River without any treatment. According to local fishermen, the industry around the station consistently discharges its waste directly into the river without any treatment. Scheibye *et al*. (2014) and Zhang *et al*. (2016) stated that metals, especially Pb in sediments with high concentrations are often measured in rivers, lakes, or reservoirs in cities and near industrial areas. Several studies have indicated that the discharge of untreated industrial wastewater and domestic sewage has been a remarkable

cause of metals entering the water system and accumulating in sediments, e.g., (Di Cesare *et al*., 2020; Dendievel *et al*., 2022).

Alternatively, Kang *et al*. (2019) reported that higher DO concentrations, i.e., DO > 5 mg/L, facilitated metal release from sediments, while lower DO, i.e., DO < 3 mg/L, enhanced metal adsorption. Standards for metals in sediments refer to the Australian and New Zealand Environment and Conservation and the Agriculture and Resource Management Council of Australia and New Zealand (ANZECC and ARMCANZ, 2000) and the United States Environmental Protection Agency (USEPA, 2004). Pb concentrations in sediments at all three stations did not exceed the standard limits of ANZECC, ARMCANZ (47.82 mg/kg), and USEPA (50 mg/kg). However, despite not exceeding the standard limits, this study demonstrated biological effects on the test organisms, indicating that the quality standards may need to be reviewed. Moreover, several biomarkers also presented effects from exposure to SS at concentrations below the standard values.

Egg Diameter

Exposure to SS at station 1 (SS 1) and station 2 (SS 2) affected the egg diameter size of *O. celebensis* embryos, increasing egg diameter size compared to ERM (control medio) (Table 1, Figure 3). According to Yaqin *et al*. (2024), the type, concentration, and length of exposure to a pollutant all affect the embryonic egg diameter and can lower the quality of the egg. Fish embryos' permeability, swelling, and water absorption are all reflected in their egg diameter. According to Liu *et al*. (2021), exposure to high Nickel (Ni) concentrations increased chorion permeability and egg diameter while low concentrations of Ni lowered egg diameter. Wang *et al*. (2020) discovered that exposure to Copper (Cu) at 0.08 to 1.28 mg/L significantly increased the egg diameter of *O. melastigma* embryos. The increase in egg diameter may result from interference from absorbed metals or contaminants, causing high osmolarity (Yokokawa *et al*., 2023). According to Jezierska *et al*. (2009), the air absorption by the perivitelline space, which contains colloidal suspensions released by the vitelline membrane, that is why fish eggs get bigger. In essence, metal ions can penetrate the egg and alter its structure while the eggshell is still porous.

Somite

Somites of *O. celebensis* embryos increased in number in phases 19 to 21 (2 dpf) in all exposure media. However, somite development in SS 2 was slower (Figure 4). Somites are crucial temporary structures for the development of the axial skeleton, spinal nervous system, and body muscles (Musumeci *et al*., 2015). Elmasri *et al.* (2004) stated that disruption of the Delta/Notch pathway inhibits the expression of oscillating genes, thereby disrupting somite formation. The Delta/Notch pathway regulates segmentation timing, somite boundary formation, and cell

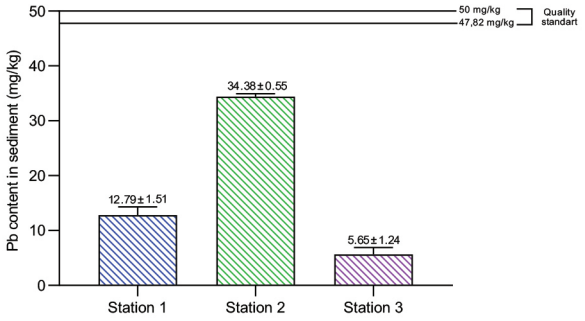


Figure 2: Concentration value of Pb content in sediment at each station. Pb content in each media does not exceed the standard value of water quality standards. Numbers indicate mean ± standard deviation (sd)

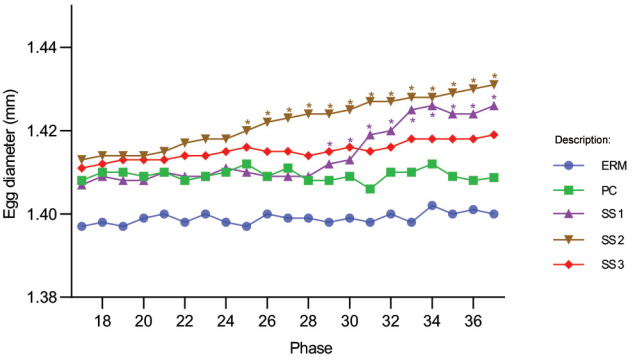


Figure 3: Egg diameter (mm) of Medaka fish (*O. celebensis*) embryo. In the ERM (control media) group (blue), egg diameter remained relatively stable overall. The egg diameter of the embryos exposed to SS 1 (purple) and SS 2 (brown) increased in size in the middle of the organogenesis phase, namely phases 24 and 29. The size of the egg diameter exposed to PC (green) and SS 3 (red) media was not significantly different from the embryos in the control media (ERM), which were relatively the same size from the beginning to the end of the exposure. Statistically significant differences from the ERM (control media) treatment ($p < 0.05$) are represented by asterisks

differentiation in Medaka fish (Naganathan & Oates, 2020). Segmentation requires physical boundary formation to prevent cell mixing. Mutations in the Delta/Notch pathway result in phenotypes that disrupt somite segmentation, emphasising the importance of this pathway in normal development (Ramesh & Chu, 2024). On the other hand, Gibb *et al.* (2010) stated that many cyclic genes are targets of this pathway, as Notch is at the core of the vertebrate segmentation mechanism. Thus, environmental pollutants such as metals can interfere with the Delta/Notch pathway during somite formation in fish embryos. Keseroglu *et al*. (2023) indicated that environmental factors can cause segmentation defects in Notch pathway genes in *Danio rerio* embryos. Furthermore, disruption of somite development can lead to abnormal embryo development until hatching into larvae. However, the significance of the Delta/Notch pathway in the somite formation process in Medaka fish embryos, especially *O. celebensis* requires further research.

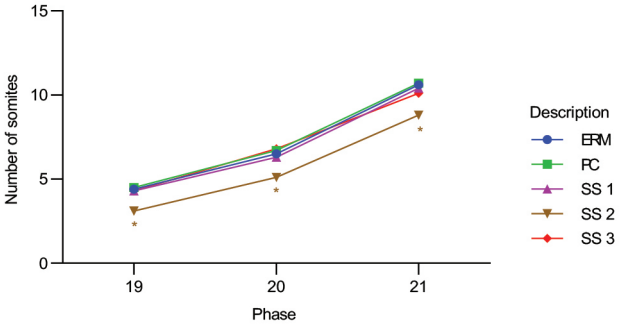


Figure 4: Somite number of Medaka fish (*O. celebensis*) embryo. Somite development continued to increase each phase in all exposure media. Embryos exposed to PC (green), SS 1 (purple), and SS 3 (red) media showed the same somite development as ERM (control media/red). Embryos exposed to SS 2 (brown) media experienced longer somite development compared to ERM media. Statistically significant differences from the ERM (control media) treatment ($p < 0.05$) are shown by asterisks

Yolk Sac Volume

Yolk volume size in *O. celebensis* embryos decreases with development as it serves as the main source of nutrients (Song *et al*., 2021). This process is known as yolk utilisation (Bik *et al*., 2020). According to Song *et al.* (2021), the yolk continues to shrink as energy transfers to the embryo's organs, with the smallest size prior to hatching (Puangchit *et al*., 2017). Embryos on SS 2 media exhibited an increase in yolk volume greater than ERM (control media) and other media (Table 1, Figure 5). This could indicate developmental problems due to environmental stress, genetic mutations, or metal toxins. Moreover, previous studies have suggested increased yolk volume due to exposure to Pb (Curcio *et al*., 2021), $MnCl_2$ (Liu *et al*., 2023), and Cu (Wang *et al*., 2020). Aldavood *et al.* (2020) noted that exposure to Cd and a combination of Ni and Cd increased the yolk sac area due to decreased metabolism. Meanwhile, Xia *et al*. (2018) stated that metals interfere with metabolism and energy production. Hence, the yolk sac remains large. At the same time, Ryuzono *et al.* (2017) mentioned an increase in yolk size in *D. rerio* embryos with a decrease in hatching embryo size.

Yolk Absorption Rate

Embryos on PC, SS 1, and SS 3 media had good yolk absorption rates, similar to ERM (control media). In contrast, embryos on SS 2 media are less able to absorb yolk well (Table 1). Yaqin *et al.* (2024) stated that nutrients from egg yolk are metabolised and absorbed by the yolk sac membrane for embryo use. Notably, the rate of yolk absorption is strongly related to the volume of yolk. Yolk absorption is essential during embryonic development as it is the main source of nutrients. In particular, low yolk absorption rates can respond to toxic xenobiotics, causing increased yolk sac and oedema (Barjhoux *et al*., 2017; Xia *et al*., 2018). Metabolic rate also affects yolk absorption. Van Leeuwen *et al.* (2017) stated that the higher the metabolic rate, the faster the embryo absorbs nutrients. Hence, metabolic disorders such as those caused by metals can inhibit nutrient absorption, causing the yolk sac to remain large (Aldavood *et al*., 2020).

Heart Rate

This study revealed that embryos exposed to PC media had almost the same heart rate as embryos in ERM (control media) media from the early (24) to late (37) phases. Embryos exposed to SS 1 and SS 2 demonstrated tachycardia from the early phase to phase 26, bradycardia in phase 27, and returned to normal in phases 34 to 37. Meanwhile, embryos exposed to SS 2 presented tachycardia until phase 27 and then bradycardia

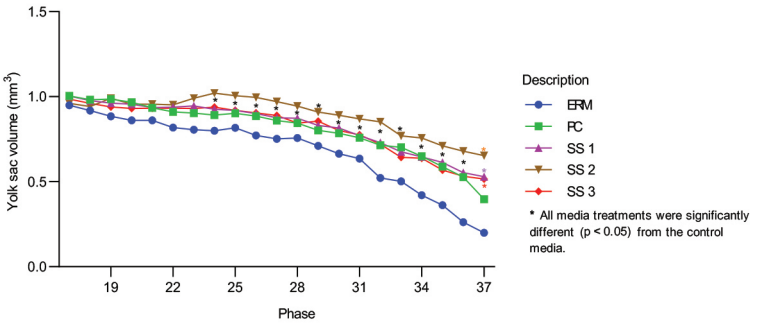


Figure 5: Yolk sac volume of Medaka fish (*O. celebensis*) embryo. Embryos in ERM media (control media; blue) show that the size of the yolk sac volume continues to decrease along with the development of the embryo. The decrease in yolk volume of embryos in PC (green), SS 1 (purple), SS 2 (brown), and SS 3 (red) media did not experience a significant decrease in each exposure medium when compared to ERM media. Embryos in SS 2 media exhibited yolk sac oedema upon entering phase 25, resulting in an increase in the yolk sac volume. Statistically significant differences from the ERM (control media) treatment ($p < 0.05$) are shown by asterisks

Table 1: Results of biomarkers value on Medaka fish (*O. celebensis*) embryo at each station

Biomarkers	Exposure Media (mean ± standard deviation)				
	ERM (control media)	PC	SS 1	SS 2	SS 3
Egg diameter (mm)	1.40 ± 0.0014	1.41 ± 0.0014	0.42 ± 0.007*	0.42 ± 0.006*	0.41 ± 0.0022
Yolk sac volume (mm^3)	0.68 ± 0.22	0.81 ± 0.16	0.83 ± 0.15*	0.9 ± 0.16*	0.82 ± 0.15*
Yolk absorption rate (mm^3/day)	0.0031 ± 0.0004	0.0025 ± 0.0003	0.0022 ± 0.0004	0.0008 ± 0.0003*	0.0024 ± 0.0005
Jaw movement (per minute)	18 ± 2.26	27 ± 1.91	0*	0*	15 ± 20.05
Hatching time (day)	10 ± 0.79	8 ± 0.87	15 ± 0.9*	16 ± 0.71*	11 ± 1,01
Survival rate (%)	100	90	60	20	90
Total length of early-hatched larvae (mm)	4.45 ± 0.009	4.45 ± 0.15	4.03 ± 0.12*	3.94 ± 0.19*	4.24 ± 0.13

Description: Statistically significant differences from the ERM (control media) treatment ($p < 0.05$) are shown by asterisks; numbers indicate mean ± standard deviation.

decreased sharply until phases 35 to 37 (Figure 6). Note that the higher the Pb concentration in the sediment, the lower the embryonic heart rate (Schweizer *et al*., 2022). According to Huang *et al.* (2023), embryonic *O. melastigma* heart rate was proven to be accelerated by a low dose of Bisphenol A Fluoride (BPAF)/ [4,4′-(Hexafluoroisopropylidene)diphenol (10 µg/L)] and lowered by a high dose (5 mg/L). Simultaneously, metals and other pollutants found in the environment can harm an embryonic heart (Boulanger *et al*., 2019), organic pollutants (Zheng *et al*., 2020), and nanoparticles (Cong *et al*., 2017), causing impaired development.

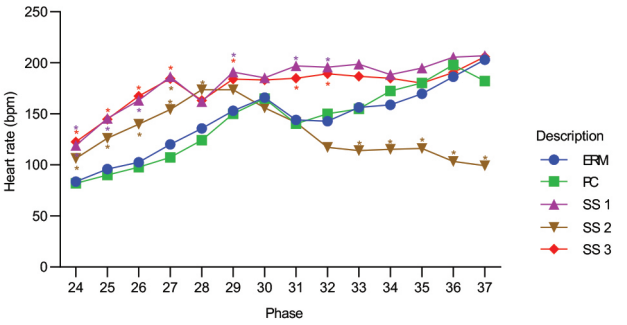


Figure 6: Heart rate of Medaka fish (*O. celebensis*) embryo. The heart rate of embryos exposed between ERM (control media; blue) and PC media (green) showed almost the same beat rhythm from the beginning of the phase to the end of the phase. The heartbeat of embryos exposed to SS 1 (purple) and SS 3 (red) media shows a faster heartbeat (tachycardia) than embryos in ERM media. Embryos exposed to SS 2 (brown) media show a different rhythm from ERM media, namely experiencing a decrease (bradycardia) in the middle to the end of the phase. Statistically significant differences from the ERM (control media) treatment ($p < 0.05$) are shown by asterisks

In addition, metals can cause cardiovascular disorders in Medaka Fish embryos, resulting in tachycardia and bradycardia. Furthermore, exposure to contaminants such as $MnCl_2$ increased the heart rate of *O. melastigma* embryos (Liu *et al*., 2023) while Silver Nanoparticles (AgNPs) slowed the heart rate in Medaka Fish embryos (Cho *et al*., 2013). On top of that, Pb-induced cardiovascular toxicity at 100 µg/L caused a decrease in heart rate (Kataba *et al*., 2022).

Jaw Movement

Jaw activity in *O. celebensis* embryos is characterised by the lower jaw opening and jaw movement. Here, embryos exposed to PC and SS 3 media demonstrated active jaw movement and did not exhibit significant differences in ERM (control media) (Table 1). Development in normal embryos in Medaka Fish begins to move its jaw when the embryo enters phase 36 (8 dpf) (González-Doncel *et al*., 2005). In contrast, embryos exposed to SS 1 and SS 2 revealed no movement when the embryos entered phase 36. Studies on the effects of contaminants on the jaw movement of Medaka fish embryos are still minimal. However, the inhibition of jaw activity is thought to be related to cardiac activity. Note that normal jaw movement is a sign of good embryonic development. Therefore, changes in jaw activity may reflect disturbances in embryonic development. Schweizer *et al.* (2022) stated that a well-developed heart is essential to ensure an adequate supply of oxygen and nutrients.

The supply of nutrients and oxygen also flows to the muscles that control the jaw. If the heart has abnormalities, the supply of oxygen-rich blood and nutrients to the jaw muscles will also be affected, inhibiting jaw activity (Lall & Lewis-McCrea, 2007). Inhibited jaw activity is also related to the production and function of hatching enzymes located in the head of the embryo. Moreover, fish embryos have hatching enzymes in the anterior region, frequently near the head and mouth. The hatching enzyme gland in Medaka Fish usually develops in the ventral part of the head (González-Doncel *et al*., 2005). Consequently, this association may cause delays in hatching time.

Hatching Time

The time it took for embryos to hatch into larvae in this investigation differed depending on the type of exposure media. Embryos exposed to PC media had the highest hatching rate, where those exposed to SS 2 had the lowest hatching rate, as indicated in Table 1. *Cong et al.* (2017) reported that the typical incubation period for *O. melastigma* embryos ranges from 10 to 14 days. Embryos that were exposed to PC exhibited early hatching, as observed in the study conducted by (Wang *et al*., 2020). The study noted that low levels of Cu impact the structure of the chorion and elevate the metabolic rate, leading to premature hatching.

Conversely, when exposed to SS 2 containing high levels of Pb, the time it took for hatching to occur was extended. This finding aligns with a previous study by Liu *et al.* (2021) that investigated the impact of Ni on the hatching of *O. melastigma* embryos. Heavy metals can modify the function of hatching enzymes, impacting the time it takes for eggs to hatch. Exposure to $MnCl_2$ (Liu *et al*., 2023) and Ni (Liu *et al*., 2021) was proven to decrease hatching enzyme activity and slow down hatching. Heavy metal toxicity can also cause embryonic death and reduce hatchability. Jaw activity is related to hatching enzymes. The enzyme chorionase, synthesised by the hatching glands located in the fish head, is essential for hatching (Fraysse *et al*., 2006; Jezierska *et al*., 2009). Note that jaw movement aids the distribution and release of enzymes around the chorion, accelerating hatching (Yamagami, 1981). Without effective jaw movement, enzymes may not be properly dispersed or activated to dissolve the chorion efficiently.

Survival Rate Embryo

The percentage of embryo survival in each exposure medium is ERM (control media) 100%, PC 90%, SS 1 60%, SS 2 20%, and SS 3

90% (Table 1). This study suggests that exposure to contaminants such as SS 2 containing Pb metal significantly impacts embryo survival rates. The findings of this study reveal a positive correlation between metal content and embryo mortality, indicating that as the metal concentration increases, so does the embryo mortality rate. Based on the studies conducted by Barhoumi *et al.* (2016), Taslima *et al.* (2022), and Macirella *et al*. (2023), it has been observed that the sensitivity of fish embryos to heavy metals varies at different stages of development. This variation is influenced by factors such as the type of fish and metal species and the metal concentration.

Furthermore, the elevated mortality rate observed in fish embryos is attributed to the presence of heavy metals. The elevated mortality rate observed in embryos exposed to SS 2 can be attributed to the abrupt cessation of blood cell activity upon entering phase 37 (8 dpf), leading to the sudden death of the embryo. The study conducted by Ismail and Yusof (2011) demonstrated that fetal development ceased immediately due to cardiovascular abnormalities when exposed to a concentration of 1.0 mg/L Cd. Therefore, the embryo exhibits a degree of tolerance when subjected to a concentration of 1.0 mg/L Cd.

Total Length of Early-hatched Larvae

The initial length of successfully hatched larvae presented differences in size in embryos exposed to SS 1 and SS 2 compared to ERM (control media) (Table 1). Both media produced larvae with shorter body lengths. Meanwhile, larval body length correlates with the heart's functional capacity and metabolic rate. Taslima *et al.* (2022) stated that fish in their early embryonic stages are utilised as bioindicators of toxicity since they are more vulnerable to contaminants than adult fish (Rahman *et al*., 2020). According to Liu *et al*. (2021), Ni exposure in *O. melastigma* embryos increased the heart and metabolic rates, resulting in shorter body-length hatchlings. Hence, short body length results from stress throughout embryonic life, which lowers energy for development (Lara & Vasconcelos, 2021).

In embryos exposed to SS 1, increased heart rate and high metabolic rate led to shorter larvae compared to ERM (control media). In contrast, in SS 2, low heart rate and yolk absorption rate resulted in short larvae with large yolk sacs. Contaminants in the Tallo River contain various pollutants that impact embryos differently (Sojka & Jaskula *et al*., 2020). Pollutant stress requires energy, diverting growth energy to compensate for lost energy. According to Yaqin *et al.* (2024), newly hatched larvae's body length correlates with their ability to absorb the yolk sac and faster heart rate. This statement is consistent with the results on larvae from ERM (control media), PC, and SS 3 media, indicating a relationship between heart rate, metabolic rate, and larval body length.

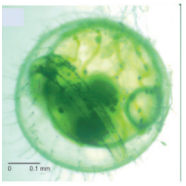
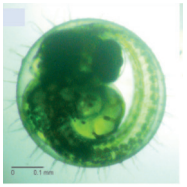
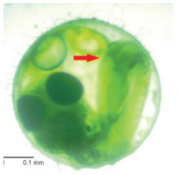
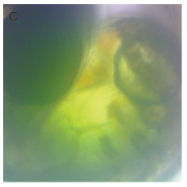
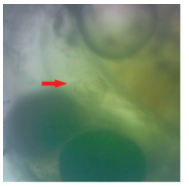
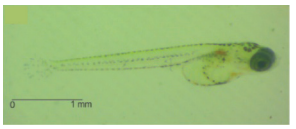
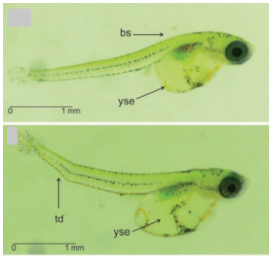
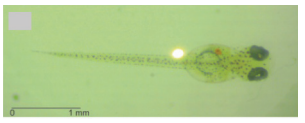
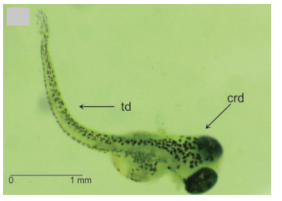
Morphology Abnormalities

Abnormalities occur in the form of morphological abnormalities in embryos and larvae (Table 2). The observed abnormalities are yolk oedema (yse), spinal deformities [backbone deformity (bs), tail deformity (td)], cardiovascular anomalies (anaemia resulting in the absence of circulating blood cells and local accumulation of immobile blood cells), and craniofacial deformities (crd). Note that deformities caused by Pb are dose-dependent. Thus, the higher the metal concentration, the higher the percentage of abnormalities that occur in the embryos and larvae.

The injury severity was significantly greater in the high-concentration group at all exposure times, proving a strong correlation between Pb dose and the incidence of morphological abnormalities. According to the statement Jezierska *et al.* (2009), Pb causes dose-dependent deformities, with high metal concentrations increasing the percentage of abnormalities.

Metal ions can accumulate and cause developmental abnormalities or even death in embryos that are not completely protected from them. Prior research has also demonstrated Pb-

Table 2: Morphology abnormalities in embryos and larvae Medaka fish (*O. celebensis*) exposed to supernatant sediment from station 2

ERM (control media)	SS 2 (supernatant sediment from station 2)	Description
		Embryos that experience tail bending at phases 26 (5 dpf)
		Embryos that experience tail bending at 35 (11 dpf)
		Embryos that experience anaemia or lack of red blood cells (erythrocytes) at phase 35 (11 dpf)
		Embryos that experience yolk oedema
		Larval morphological abnormalities observed were tail deformity (td), dorsal deformity (bs), craniofacial deformity (crd), yolk oedema (yse) (17 dpf)
		

Description: Red arrows indicate abnormalities in the embryo, dpf: day post-fertilisation.

induced abnormalities in fish embryos, larvae, and other metals (Barjhoux *et al*., 2017; Cong *et al*., 2017; Yin *et al*., 2017). The most prevalent abnormalities reported are spinal ones, which are brought on by early-stage defective somite development. Embryos exposed to SS 2 develop yolk oedema due to a decreased metabolic rate, which lowers nutritional use (Sfakianakis *et al*., 2015). Meanwhile, cardiovascular anomalies such as anaemia and accumulation of immobilised blood cells, lead to high mortality rates in SS 2 embryos. About 80% of embryos develop immobilised red blood cells in phase 36 (8 dpf), causing sudden death (Macirella *et al*., 2023).

Conclusions

This study demonstrates the potential for all of the straightforward biomarkers included in this research to identify pollutants in contaminated waters. Using a statistical method, the endpoint results demonstrated the ability to identify contaminants in the Tallo River's sediments. The tests implied that exposure to contaminants from the Tallo River sediments exerted various effects on *O. celebensis* embryos and also resulted in defective development. Although the reference concentration of Pb contained in the sediment has not exceeded the quality standard limit, the research study's results demonstrated the disruption of the biological development of the test animals. This could reflect that although Pb concentrations are still below the established safe limits, long-term exposure or accumulation of Pb in sediments in the ecosystem could still potentially cause adverse effects on organisms. Nevertheless, this indicates the need for further review of existing quality standards as well as the importance of continuous monitoring to prevent adverse environmental impacts.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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