

DETECTION OF TRICLOSAN POLLUTANTS ADSORBED ON POLYPROPYLENE MICROPLASTICS USING SIMPLE BIOMARKERS IN THE EMBRYOS OF *Oryzias javanicus* (BLEEKER, 1854)

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Abstract: Microplastics are one of the major pollutants with the ability to adsorb other pollutants such as triclosan. This potentially makes them vectors for the spread of triclosan in aquatic environments. This study analyses *Oryzias javanicus* embryo biomarkers that are sensitive for detecting the triclosan adsorbed on polypropylene microplastics. The treatments used are Treatment A, using Embryo Rearing Medium (ERM) as control, Treatment B, using ERM and Dimethyl Sulfoxide (ERM+DMSO), Treatment C, using triclosan solution (TCS), Treatment D, using polypropylene microplastics (PP), and Treatment E, using mixture of polypropylene microplastics and triclosan (PP+TCS). The observed biomarkers included number of somites, heart rate, yolk absorption rate, hatching time, total body length of the newly hatched larvae, and embryo survival rate. The results showed a significant difference ($P < 0.05$) in heart rate, hatching time, total body length of the newly hatched larvae, and embryo survival rate for those exposed to PP+TCS. However, other biomarkers did not show a significant difference ($P > 0.05$). This study suggests that heart rate, hatching time, and total body length of the newly hatched larvae of *O. javanicus* embryos have the potential to be used as biomarkers for effect-based biomonitoring.

Keywords: Biomarker, *Oryzias javanicus*, embryo, microplastic, triclosan.

Introduction

Plastic plays a crucial role in daily life, as evident in its prevalence in single-use medical equipment, food packaging, and various technologies (Ricciardi *et al.*, 2021). However, plastic is often disposed of into the environment. Major categories of plastic waste include industrial waste, medical waste from hospitals, litter from tourist areas, and domestic waste. Plastic disposed of into the environment will undergo physical and chemical breakdown over time into smaller particles due to degradation. Plastic particles with a size ranging from 0.1 μm to 5 mm are referred to as microplastics (Gupta *et al.*, 2022; Syranidou & Kalogerakis, 2022).

Microplastics are one of the main pollutants in both freshwater and marine environments, posing a significant threat to aquatic ecosystems and biodiversity (Gupta *et al.*, 2022). The presence of microplastics in water is continuously increasing due to their non-degradable nature, leading to accumulation

and the adsorption of other pollutants on the surface of microplastics (Gupta *et al.*, 2022). The persistent nature of microplastics, which are resistant to natural degradation processes, contributes to their long-term presence in aquatic environments. Microplastics also act as vectors in adsorbing other chemical pollutants and persistent organic pollutants carried by water on the surface of microplastics, which can elevate the risk of microplastic pollution (Kabir *et al.*, 2021). Once detached, microplastics can be ingested by organisms through various pathways, such as contaminated water or consumption of contaminated organisms. The contaminants associated with microplastics may leach into tissues, leading to potential bioaccumulation as they move up the food chain (Sarijan *et al.*, 2021).

The COVID-19 pandemic brought attention to one source of microplastics: Disposable face masks (DFMs). Studies have found that DFMs

release a significant amount of microplastics into the environment, both on land and in water (Fadare & Okoffo, 2020). The COVID-19 pandemic has led to a surge in disposable face mask waste, which is now recognised as a new contaminant source. The widespread use of face masks as a preventive measure has significantly increased the generation of single-use mask waste, presenting environmental challenges and potential health risks (Sullivan et al., 2021). According to Chen et al. (2021), loose-bound plastic fibres in disposable face masks are a significant source of microplastic release. The majority of single-use face masks are made from various polymers, including polypropylene (PP), polyurethane (PU), polyacrylonitrile (PAN), polystyrene (PS), polycarbonate (PC), polyethylene (PE), and polyethylene terephthalate (PET) (Chen et al., 2021). PP fibre is the most commonly used material in face mask production (Ma et al., 2021).

Microplastics have the ability to adsorb other pollutants, including antibiotics (Atugoda et al., 2021), making them potential vectors for the spread of antibiotics in water (Zhang et al., 2020). Research by Lin et al. (2022) explains that polypropylene (PP) plastic fibres are a major component of disposable face mask layers that can adsorb triclosan (TCS) through hydrophobic interactions. Triclosan is one of the antibacterial agents widely used in personal care products such as toothpaste, soap, deodorant, cosmetics, and skincare lotions (Yuan et al., 2020). The COVID-19 pandemic has led to a significant increase in demand for disinfectants. This has resulted in a rise in triclosan productivity, which in turn has led to urban waste entering water bodies (Yin et al., 2022). Triclosan is persistent, remaining in the environment for an extended period (Wang & Liang, 2021). Triclosan is persistent and as a hydrophobic and lipophilic organic chemical, it can dissolve through the soil, leading to groundwater pollution and persisting in the environment for an extended period (Wang & Liang, 2021). Several studies have demonstrated the effects of polypropylene microplastics and triclosan (Lei et al., 2018;

Stenzel et al., 2019; Ma et al., 2020; Song et al., 2020).

Exposure to polypropylene damages the intestines of zebrafish, such as villi and enterocyte impairment (Lei et al., 2018). Triclosan exposure can alter reproductive behaviour of zebrafish, leading to a decrease in fecundity (Stenzel et al., 2019). Embryos of *Oryzias latipes* exposed to triclosan exhibit morphological abnormalities in the early stages of life (Song et al., 2020). Ma et al. (2020) found that combined exposure to polypropylene and triclosan results in higher triclosan accumulation and worsens the metabolic condition of zebrafish compared to exposure to polypropylene or triclosan alone.

Until now, there has been no research on the detection of triclosan adsorbed on polypropylene microplastics, particularly using biomarkers in model organisms like embryos. Biomarkers are biological responses of an organism to environmental stress (Yaqin, 2019). Biomarkers are highly sensitive to very low concentrations of stressors and their fluctuations can be monitored and compared with normal ranges. This approach is a robust and cost-effective method for obtaining information about the environmental conditions and the impact of pollution on biological resources (Rudneva, 2013).

The use of biomarkers in embryos is chosen because the embryonic stage is the most vulnerable or sensitive (Chen et al., 2020), thereby expected to provide a rapid biological response to environmental stress. One excellent and potentially significant alternative in vivo model is the embryos of the *Oryzias javanicus* fish (Merino et al., 2020). *Oryzias javanicus* fish is a local Indonesian species with several advantages compared to other model animals such as being productive, having no limitations in spawning season, and reaching maturity in only two to three months (Lin et al., 2016). They have large and transparent eggs, making embryo development and biomarkers such as somite count, heart rate, yolk sac, and others easily observable under a microscope, generating

less waste when using embryos, and exhibiting individual responses (Puspitasari, 2016; Yaqin, *et al.*, 2021).

Therefore, this research is necessary to analyse the effects of triclosan adsorbed on polypropylene microplastics using *Oryzias javanicus* embryos, the biological responses of which can serve as simple and sensitive biomarkers. The embryonic stage of *Oryzias javanicus* is sensitive to pollutant exposure, making it suitable for effect-based biomonitoring.

Materials and Methods

This research was conducted from June to September 2023, with the sampling location in the Jeneberang River, Makassar (Figure 1). Laboratory tests were carried out at the Aquatic Animal Physiology Laboratory, Department of Fisheries, Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar. The tools and materials used in this study were: Net, aerator, aquarium, petri dish, microplate, trinocular microscope, optilab microscope camera, deck glass, object glass, blender,

WiseShake, filter with a pore size of 5 mm, and laminar flow. The materials used were: *O. javanicus* fish eggs as the observation object, Embryo Rearing Media (ERM), Dimethyl Sulfoxide (DMSO) consisting of 10 g NaCl, 0.3 g KCl, 0.4 g CaCl, 2 mL H₂O, 1.63 g MgSO₄ mixed with 1 mL NaHCO₃ (0.2 g/20 mL H₂O), Otohime B1 pellets, and Artemia as the feed for *O. javanicus* fish, polystyrene microplastics and triclosan as pollutants in *O. javanicus* embryos.

Collection of Breeding Stock from the Wild

The fish were captured using a net with a 2 m size and a mesh size of 3 mm. The number of fish caught was 50. The captured fish were then placed in plastic containers equipped with a portable aerator and transported to the laboratory for further cultivation. During the transport, the fish were secured in the plastic containers and a suitable environment for their well-being was ensured. A portable aerator was used to maintain adequate oxygen levels in the water to preserve the health of the fish. Once the fish were securely housed in the plastic containers with the aerator, they were transported to the laboratory. This transportation phase aimed to minimise stress



Figure 1: Map of sampling location
Source: Google satellite (2020)

on the fish and provide optimal conditions for their survival.

Maintenance of Breeding Stock in the Laboratory

After the fish samples arrived at the laboratory, acclimatisation was conducted. The water temperature inside the bag or plastic containing the fish was equated with the water temperature in the aquarium. The fish were placed in aquariums and fed with commercial feeds, specifically Otohime B1 and Artemia. The appropriate amount of feed was provided, which was 5% of their body weight. Otohime B1 feed was given twice a day and Artemia was provided once a day. To maintain a healthy environment for the fish, it was crucial to clean the aquarium when leftover feed accumulated at the bottom. Cleaning helped prevent the buildup of uneaten feed and ensured the water quality remained suitable for the well-being of the fish.

Preparation of *Oryzias javanicus* Embryos

Fertilisation in *O. javanicus* fish occurred naturally in the aquarium. The female breeders that had laid eggs were removed from the aquarium using a net and the eggs were carefully extracted and immediately transferred to a petri dish filled with ERM solution. The separation of the eggs from their filaments was performed by gently rotating the eggs using the index finger until they were separated from each other.

The separated eggs were then observed under a microscope at a magnification of 40x to select the eggs to be used. The selected eggs were those that had been fertilised, indicated by the presence of the perivitelline space (Yaqin *et al.*, 2021) and had not yet passed stage 17. The embryo's development followed the observations made by González-Doncel *et al.* (2005), who conducted embryogenesis studies on *O. latipes* fish.

Preparation of Microplastics and Triclosan (TCS)

The mixing of polypropylene microplastics and triclosan in this study were modified from

the adsorption experiments by Wang *et al.* (2021), involving the combination of 200 µg/l polypropylene microplastic particles from single-use face masks and the experiments by Song *et al.* (2020), with a triclosan solution of 0.02 mg/l. The blending of microplastics and triclosan will be carried out using WiseShake at a speed of 180 rpm for 96 hours (Sheng *et al.*, 2021).

The preparation of microplastic samples from single-use face masks involved cutting the layers of the face mask into small pieces, which were then grounded in a blender. After grinding, the mixture was passed through a size of 5 mm filter. The 0.02 mg/l triclosan solution used was obtained by preparing triclosan dissolved in 0.016% DMSO (Song *et al.*, 2020).

Experimental Design

The study was conducted with a quasi-experimental design, consisting of four control treatments and one actual treatment, each with 10 replications (each representing 1 egg). The treatments included Treatment A, which is Embryo Rearing Medium (ERM), Treatment B, which is ERM and DMSO (ERM+DMSO), Treatment C, which is triclosan solution (TCS), Treatment D, which is polypropylene microplastics (PP), and Treatment E, which is a mixture of polypropylene microplastics and triclosan (PP+TCS). Treatments A and B served as controls, Treatments C and D as positive controls, and Treatment E as the actual treatment.

Subsequently, each pollutant from each treatment was introduced into a 24-well microplate using a dropper pipette. Each well of the microplate was filled with 2 ml of the prepared solution. Each well of the microplate was then populated with one selected egg. Therefore, the number of eggs used for each treatment was 10. The total number of eggs to be observed was 50, obtained from the breeding of the parent fish in the aquarium. The embryos were exposed to pollutants from neurula stage (stage 17) to stage 37 or until hatching. In their natural habitat, eggs carried by *Oryzias* parents

are directly exposed to pollutants in the aquatic environment. The direct exposure to the prepared material in this study was intended to replicate conditions in the natural habitat of *Oryzias* embryos, where eggs carried by *Oryzias* parents are directly exposed to contaminants in the water environment. This aligns with findings from Yaqin *et al.* (2024), which demonstrated that contaminants in the Tallo River water resulted in a reduction in somite count and changes in heart rate in *Oryzias celebensis* embryos.

Parameter Observed in the Study

Embryo parameters measured were number of somites, heartbeat, heart size, yolk absorption rate, hatching time, total body length of the newly hatched larvae, and survival rate.

- (1) Number of somites: The number of somites was counted directly from images captured using a microscope. The number of somites was counted at stages 19 to 21.
- (2) Heart rate and heart size: Heart rate was measured by recording the time taken to reach 30 beats. The time to reach 30 beats for each fish embryo is converted into the number of heartbeats per minute using the formula by Chen *et al.* (2020).

$$N = 30/T \times 60 \tag{1}$$

where:
 N = the number of heartbeats per minute (beats/minute)
 T = the time taken to reach 30 beats

- (3) Yolk sac absorption rate
 The absorption rate of yolk is calculated using the formula by Dharma (2015).

$$YS_{AR} = \frac{Vo - Vt}{T} \tag{2}$$

where:
 YS_{AR} = Yolk sac absorption rate (mm³/hour)
 Vo = Initial yolk sac volume (mm³)
 Vt = Final yolk sac volume (mm³)
 T = Time (hours)

- (4) Hatching time: Observations of hatching time were conducted by noting the rupture of the chorion membrane of the eggs and the active movement of the larvae. Hatched eggs were then recorded daily, starting from the first day of hatching until all embryos in each maintenance medium hatched. Only embryos that were able to fully emerge from the chorion were considered as hatched while others were considered as not hatched (Wang *et al.*, 2020).
- (5) Total body length of the newly hatched larvae: Observation of the larvae were conducted using a stereo microscope with a magnification of 1.5x. The images of the larvae were then documented using the Optilab application. The total body length of all newly hatched larvae were measured using the Image Raster 3.0 application. The length of the larvae was measured each time an embryo hatched in each treatment.
- (6) Survival Rate: The survival rate of embryos was counted based on the formula by Siddique *et al.* (2023).

$$SR = \frac{N_t}{N_o} \times 100\% \tag{3}$$

where:
 SR = Survival rate
 N_t = Number of the live embryos at the end of the study
 N_o = Number of embryos at the start of the study (10 embryos per treatment)

Data Analysis

Statistical analysis was performed using GraphPad Prism 8 software, employing the non-parametric statistical test, the Kruskal-Wallis test to analyse the comparison of number of somites, yolk sac absorption rate, heart rate, hatching time, and total body length of the newly hatched larvae. The results of the Kruskal-Wallis test were followed by Dunn Multiple Comparison test for analysing the comparison of each treatment. The data on embryo survival rate were analysed using descriptive analysis.

Results and Discussion

Number of Somites

Somites are segmented blocks of mesoderm in vertebrate embryos that form the basis for the development of segmented body structures during embryonic development (Piatkowska *et al.*, 2021). In this study, the somites of *O. javanicus* embryos showed no significant differences between those treated and the control group. The number of somites in each treatment corresponded to the somite count during the normal development of *O. latipes*, as reported by González-Doncel *et al.* (2005), indicating the development of two to five somites at stage 19 and five to eight somites at stage 20.

The polypropylene microplastics and triclosan in this study did not have an effect on the somite count of *O. javanicus* embryos. While this finding indicated that somite development in *O. javanicus* embryos was not affected by the presence of polypropylene microplastics and triclosan, it is important to note that these substances may still have an impact on other developmental or physiological aspects. Similar findings were also reported in a study by Oliveira *et al.* (2009), where triclosan at 0.9 mg/l did not affect the number of somites of zebrafish embryos. The toxins are expected to initially interact with the chorion of the embryos, acting as a barrier that separates the internal and external environment of the embryos. The chorion is a protective membrane that surrounds

the developing embryos of many aquatic organisms, including fish and amphibians. It serves as a crucial interface that regulates the exchange of substances between the embryo and its surroundings (Concha & Reig, 2022). The chorion surrounds the oocyte and plays a crucial role in fish fertilisation. It shields the developing embryo from environmental and mechanical factors until hatching. Choriogenins, a group of glycoproteins, make up the chorion and their synthesis is regulated by the plasma concentrations of the estradiol-17 β hormone (Pérez-Atehortúa *et al.*, 2023). The comparison of the number of somites of each treatment group can be seen in Figure 2.

There were no significant differences ($P > 0.05$) in each treatment during stages 19 to 21. At stage 19, the average somite count for the ERM treatment is four somites, DMSO has five somites, TCS has five somites, PP has four somites, and PP+TCS has four somites. At stage 20, there was an increase in the number of somites. The average somite count for ERM was seven somites, DMSO was seven somites, TCS was seven somites, PP was six somites, and MP+TCS was seven somites. At stage 21, the somite count further increased. The average somite count for the ERM treatment was 10 somites, DMSO had 11 somites, TCS had 11 somites, PP had 10 somites, and PP+TCS had

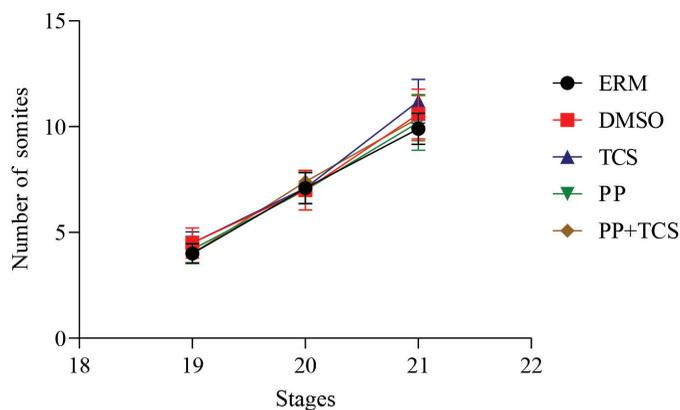


Figure 2: The number of embryo *Oryzias javanicus* somites were incubated in treatment group

10 somites. Fish from the *Oryzias* genus have a thick and hard chorion that functions to hinder the diffusion of unwanted molecules. The thickness and stiffness of the *Oryzias* fish chorion play a key role in limiting the entry of unwanted molecules, including potential toxins or contaminants into the embryo (Tazin *et al.*, 2023). This protective function helps shield the developing embryos from environmental threats, contributing to the survival and normal development of the fish during the early stages of their life cycle. Therefore, if molecular diffusion into the embryos occurs, the process may proceed at a slow pace (Yaqin *et al.*, 2021).

The *O. javanicus* embryos exposed to triclosan and microplastics were protected by a thick and hard chorion, preventing the effects of triclosan and microplastic exposure during 10 hours period (stages 19 to 21) from causing any significant impact leading to apoptosis of cells that would affect the normal somite count (De Guzman *et al.*, 2020). This indicates that microplastics and triclosan in this study did not influence the somite count and therefore, could not be used as biomarkers. The absence of an effect on the number of somites indicates that this developmental parameter may not be sensitive to the presence of microplastics and triclosan in the tested concentrations or conditions.

The type of microplastic used in this study was unable to penetrate the chorion pores and enter the egg. Similarly, the concentration of ERM, DMSO, TCS, PP, and PP+TCS were also unable to diffuse into the embryo in this stage. It is suspected that if these pollutants could diffuse into the embryo, the amount that actually entered would be minimal due to the short exposure period and the early stage, when the chorion is still very thick. Therefore, each treatment did not have a significant effect.

Heart Rate

Other organs vulnerable to environmental stress are described in the research by Chen *et al.* (2022), particularly the heart, which is one of the first organs to undergo organogenesis and environmental pressure can lead to abnormal

embryonic heartbeats. An alteration in heart rate may be indicative of physiological stress or developmental abnormalities due to exposure to contaminants. Increased or decreased heart rate may indicate possible cardiotoxic effects, which may have wider consequences on the viability and health of the developing organism (Ibrahim *et al.*, 2020). Changes in the heart rate of *Oryzias* embryos serve as valuable indicators of embryonic toxicity, providing insights into the adverse effects of contaminants (Yaqin *et al.*, 2021).

In this study, we found that there was a significant difference ($P < 0.05$) in the TCS treatment at stages 24, 25, 27, 33, 34, 36, and 37, as well as in the PP+TCS treatment at stages 26, 27, 29, 33, 36, and 37 compared to the control. Stages 36 and 37 are considered the final stages, representing the accumulation stages of TCS and PP+TCS from previous stages. In these stages, *O. javanicus* embryos exposed to TCS and PP+TCS experienced a decrease in heart rate. Meanwhile, the DMSO and PP treatments did not show a significant difference ($P < 0.05$) compared to the control (Figure 3).

The current study found that *O. javanicus* embryos exposed to TCS experience tachycardia (increased heart rate) in the early stages. The heart rate of *O. javanicus* embryos exposed to DMSO and PP remained normal from the early to the late stages. In contrast, embryos exposed to PP+TCS exhibited decreased heart rate in the early stages. Subsequently, the heart rate of embryos exposed to TCS and PP+TCS returned to normal in the mid-stage and experienced increased heart rate in the late stages (36 to 37). These findings also suggest that heart rate in the final stages needs to be considered when using it as a biomarker for detecting pollutants.

TCS can interact with β -Adrenergic Receptors (β -AR) in embryonic hearts (Wan *et al.*, 2023). This interaction suggests that TCS may impact the function of β -Adrenergic Receptors, which play a crucial role in regulating heart rate and cardiovascular responses. Stimulation of β -AR typically results in an increased heart rate. However, excessive or prolonged exposure to

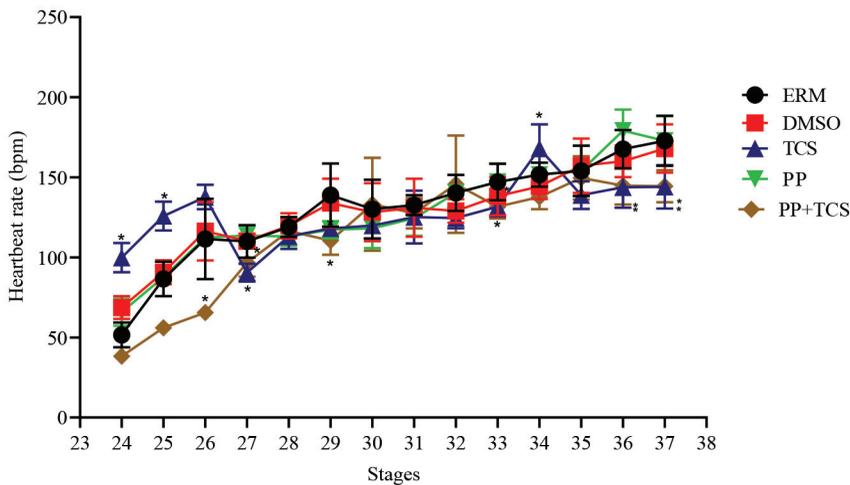


Figure 3: Heartbeat rate of embryo *Oryzias javanicus* were incubated in treatment group

toxicants can cause a slow response of the heart muscle and a decrease in heart rate (Maciag *et al.*, 2022). The study by Wan *et al.* (2023) exposing zebrafish embryos to 1.4 μM TCS resulted in a significant decrease in heart rate compared to the control.

Furthermore, a study by Zhu *et al.* (2018) states that TCS and TCS metabolites can activate the Aryl hydrocarbon Receptor (AhR), which can result in cardiovascular disturbances, such as a slow heart rate. This finding is in line with the results of the study, where the heart rate of *O. javanicus* embryos increased in the early stages (tachycardia) but decreased in the later stages (bradycardia). In the early stages, the increased heart rate (tachycardia) may be linked to specific responses triggered by AhR activation. However, as development progresses, the decreased heart rate (bradycardia) could indicate a more complex and potentially adverse impact on the cardiovascular system. The findings suggest that exposure to TCS, through AhR activation, indicate that exposure to TCS, through AhR activation may contribute to disruptions in cardiac function. It is important to understand the molecular mechanisms involved (He *et al.*, 2021). Meanwhile, embryos exposed to PP+TCS experienced bradycardia in both early and late stages. This indicates that the adsorbed TCS on PP in this study affects the

heart rate of embryos. The adsorption of TCS on PP is evidenced by the change in heart rate in embryos exposed to PP+TCS, as well as to TCS alone but not in the PP treatment. This proves that in this study, TCS is adsorbed onto PP.

Even if individual contaminant concentrations are below the toxicity threshold, the combination of these contaminants can still pose health risks. Microplastics that adsorb triclosan also have the potential to release it, acting as a source and carrier of TCS (Chen *et al.*, 2021). This indicates that triclosan, once adsorbed onto microplastics has the potential to be released back into the surrounding environment. Additionally, the descriptive data suggest that the yolk absorption rate in embryos exposed to TCS and PP+TCS was lower when compared to the control group. This observation suggests that exposure to TCS and the combination of polypropylene and TCS may reduce the metabolism of embryos, which is indicated by a weakened heart rate in embryos during the late stages. This pattern is consistent with the previously noted decrease in heart rate, indicating a potential correlation between metabolic processes and cardiovascular effects. Further exploration of the relationships and underlying mechanisms is crucial for a comprehensive understanding of the physiological impacts of these toxicants on embryonic development.

Yolk Sac Absorption Rate

Toxins can alter the nutrient utilisation rates in embryos (Gupta *et al.*, 2019). This has significant implications for developing embryos in terms of nutrient availability and metabolism. Embryos have higher metabolic needs and exposure to toxicants may deplete the egg yolk prematurely. If the absorption or quantity of egg yolk is hindered, the utilisation of yolk may be disrupted, leading to a state of nutritional deprivation in the environment. If the absorption or transport of yolk is impaired or inadequate, disturbances in yolk utilisation may be indicative of metabolic conditions (Sant & Timme-Laragy, 2018).

The statistical test for the rate of yolk absorption indicated no significant difference ($P > 0.05$) between all treatments and the control. This suggests that pollutants, namely microplastics and triclosan in the study do not affect the utilisation of stored energy in the yolk sac. The average of yolk sac absorption rate in the ERM was 0.00189 mm³/hour, in the DMSO it was 0.00179 mm³/hour, in the TCS it was 0.00168 mm³/hour, in the MP it was 0.0019 mm³/hour, and in the PP+TCS it was 0.00157 mm³/hour. The comparison of yolk sac absorption rate of each treatment group is shown in Figure 4.

However, the yolk absorption in this study can be associated with and supports the results of decreased embryonic heart rate and shorter length of the initial hatching larvae. In fish, the theoretical body length of newly hatched larvae is considered a projection of the metabolic rate (yolk absorption) and heart rate (Kamler, 2008).

In this study, embryos exposed to PP and PP+TCS showed reduced yolk absorption, slower heart rates, and shorter body lengths. Lower yolk absorption indicates reduced energy utilisation. This is because slower heart rates tend to lead to lower energy expenditure. As a result, larvae hatch with shorter lengths, presumably because energy is diverted to combat pollutants and embryos conserve energy for their survival until hatching. Research by He *et al.* (2020) demonstrated that pharmaceuticals such as propofol can induce neurotoxicity in zebrafish embryos by inhibiting the electron-transport chain. This inhibition causes mitochondrial dysfunction, resulting in decreased energy production.

Hatching Time

Embryonic development and hatching are important toxicity markers in fish life history

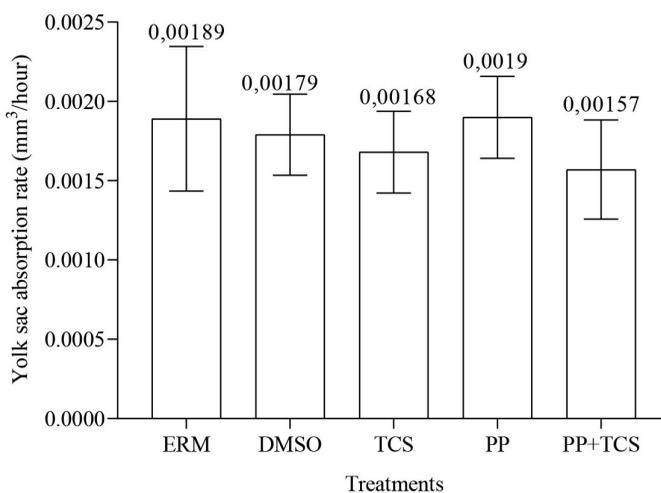


Figure 4: Yolk sac absorption rate of embryo *Oryzias javanicus* were incubated in treatment group

because they are highly influenced by water pollutants (Dar *et al.*, 2019). The hatching process in *Oryzias* involves two steps: Chorionic swelling caused by High Choriolytic Enzyme (HCE) and the breakdown of the swollen structure by Low Choriolytic Enzyme (LCE) and pactacin enzyme. The coordinated action of these three enzymes makes the hatching time and success of *Oryzias celebensis* embryos potentially useful as biomarkers for ecotoxicological studies (Yaqin *et al.*, 2022). Hatching can also be inhibited or delayed, possibly due to a decrease in motility (Wang *et al.*, 2020).

In this study, the average hatching time for embryos in the ERM treatment was 17 days, in DMSO it was 25 days, in TCS it was 26 days, in PP it was 12 days, and in PP+TCS it was 32 days. The study found that *O. javanicus* embryos exposed to DMSO, TCS, and PP+TCS had a slower hatching time compared to the control (Figure 5).

Based on Iwamatsu (2004), *Oryzias* fish eggs develop and hatch within 10 days at 26°C. In this study, embryos exposed to ERM hatched in 17 days while those exposed to PP hatched in 12 days. Although the hatching times were not significantly different, embryos exposed to PP tended to hatch faster than those exposed to ERM. This phenomenon is likely attributed to a bet-hedging strategy, where embryos exposed to ERM may adopt this strategy in response to unpredictable natural variations in their

habitat, which can influence their life history traits and maximise fitness across generations (Lips, 2001). This strategy is not only a response to environmental uncertainty but also a characteristic inherent to the organisms. Such variability emerges not only in uncertain environmental conditions but can also occur even in optimal environments.

The study by Hoyberghs *et al.* (2021) found that all embryos hatched upon exposure to DMSO at a concentration of 0.01%, which is consistent with the findings in this study. However, in this study, although all embryos hatched, there was a delay in hatching time. The reasons behind why DMSO can delay hatching time are not clear. DMSO is a solvent that interacts with the plasma membrane, forming pores that decrease membrane selectivity and increase cell permeability (de Abreu Costa *et al.*, 2017). This interaction may cause disturbances in normal development and hatching processes.

In this study, a delay in the hatching of *O. javanicus* embryos also occurred in embryos exposed to TCS and PP+TCS. Tang *et al.* (2022) found that exposure to TCS at 900 ng/l delayed the hatching of zebrafish. Research by Dar *et al.* (2019) found that the success of hatching depends on the combined action of hatching enzymes secreted by embryonic hatching gland cells and the mechanical strength generated by embryonic movement. Osmotic disturbances caused by stress on the eggs and the interaction

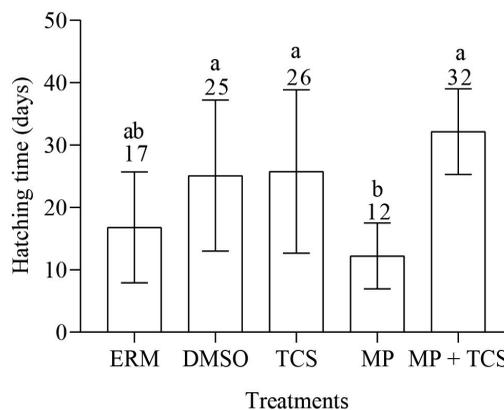


Figure 5: Hatching time of embryo *Oryzias javanicus* were incubated in treatment group. The different letters indicate a statistically significant difference from the control treatment ($P < 0.05$)

of toxins with the enzyme's active sites have been reported to disrupt or alter the activity of these enzymes (Dar *et al.*, 2019).

In this study, it was found that *O. javanicus* exposed to TCS and PP+TCS exhibited weak jaw movements compared to the control. This is related to the bradycardia condition experienced by embryos exposed to TCS and PP+TCS. The heart is part of the cardiovascular system responsible for circulating blood throughout the body, providing the necessary oxygen for various organs and tissues (Denvir *et al.*, 2008). Therefore, if the heart is unable to pump blood strongly enough, the organs will not receive an adequate supply of blood and oxygen. This condition can lead to organ failure and impaired bodily functions.

This indicates that exposure to the contaminants may disrupt the secretion of hatching enzyme. The hatching enzyme is located in the jaws of *O. javanicus* embryos, specifically in the hatching gland on the surface inside the pharyngeal cavity of *O. javanicus* embryos (González-Doncel *et al.*, 2005). Disruption of the hatching enzyme activity delays hatching of *O. javanicus* embryos. The hatching enzyme is crucial in facilitating the release of the embryo from its protective eggshell by breaking down the eggshell's structural components (Fu *et al.*, 2021). The delayed hatching time is also considered an adaptive strategy in embryos known as bet-hedging, which is an adaptive strategy for embryos to increase the chances of survival in an uncertain or changing environment (Mercer, 2021).

It is important to note that TCS primarily undergoes surface adsorption on PP, making it susceptible to desorption from the surface into local tissues (Chen *et al.*, 2021). Polypropylene microplastic significantly enhances the toxicity of TCS, even if PP does not enter the target tissues (Sheng *et al.*, 2021).

Total Body Length of the Newly Hatched Larvae

In fish, the length of newly hatched larvae theoretically reflects the rate of metabolism

(yolk absorption) and heart rate (Kamler, 2008). The higher the metabolism and heart rate in embryos, the longer the total length of newly hatched larvae will be and vice versa (Wang *et al.*, 2020). In this study, we found that the average length of larvae in the ERM treatment was 4.929 mm, in the DMSO treatment it was 4.805 mm, in the TCS treatment it was 4.855 mm, in the PP treatment it was 4.586 mm, and in the MP+TCS treatment it was 4.589 mm. This aligns with the findings of this study that the low yolk absorption in embryos exposed to PP+TCS compared to the control indicates a low metabolism in *O. javanicus* embryos, resulting in a shortened length of newly hatched *O. javanicus* larvae (Figure 6).

Additionally, many organisms exhibit adaptive life history strategies when exposed to pollutants by reducing body size to allocate energy for potential development and reproduction in the future (Wang *et al.*, 2021), which is consistent with the findings of this study. Several studies have also shown the adsorption effects of microplastics on other toxins. For instance, a study by Zhang *et al.* (2020) found that the combination of microplastics and cadmium reduced the body length of zebrafish larvae. Wang *et al.* (2021) explained that microplastics accumulated in the chorion of *O. melastigma* embryos, leading to a reduced hatching rate and the length of larval bodies. Additionally, a study by Yang *et al.* (2020) discovered a significant reduction in the body length of larvae in groups exposed to a combination of PSMP (50 ng/mL) and F-53B (10 ng/mL). Meanwhile, embryos exposed to PP also have smaller body sizes compared to the control. This is likely due to the hatching time in the PP treatment, which descriptively is faster than the control, resulting in shorter larval sizes compared to the control. According to the study by Laurel *et al.* (2008) larvae that hatch early tend to be smaller than those hatching later.

Embryos exposed to PP+TCS may potentially employ adaptive strategies to enhance their chances of survival such as reducing their body size and employing bet-hedging strategies. Asynchronous hatching is

considered an evolutionary strategy in fish and other taxa as a means of bet-hedging against dynamic variations. The bet-hedging ensures that some of their offspring can survive in a variable food environment, either by having larvae capable of feeding immediately after hatching or possessing sufficient endogenous reserves to survive until food becomes available (Laurel *et al.*, 2008). Additionally, morphological changes in the craniofacial region may result from disruptions in central nervous system (CNS) development, including abnormalities in somite organisation, neural crest cell migration, and/or the specification of normal segmental identity (Kim *et al.*, 2018).

Survival Rate

The survival of embryos is the percentage of embryos that survive until hatching into larvae. After the embryos pass the stages of embryogenesis, they will hatch. In this study, it was found that the survival rates for ERM and DMSO were 100%, TCS was 80%, PP was 90%, and the PP+TCS treatment was 70% (Figure 7). This means that there were embryos that did not hatch or failed to survive in the PP, TCS, and PP+TCS treatments. The embryos in the PP+TCS treatment exhibit the lowest survival rate compared to other treatments. Embryo death is defined as the absence of a heartbeat, which indicates the cessation of the embryonic

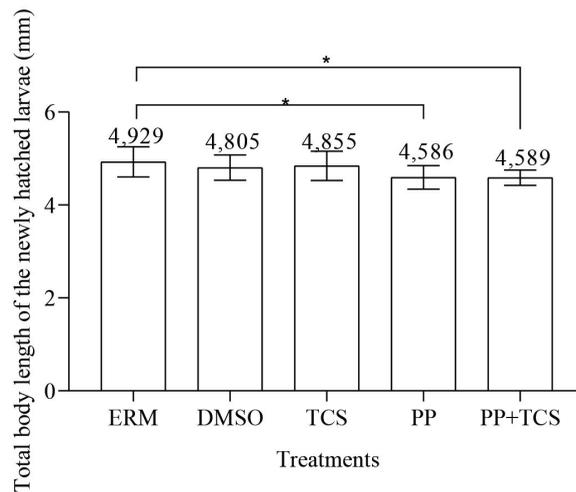


Figure 6: Total body length of embryo *Oryzias javanicus* were incubated in treatment group. The asterisk (*) indicates a statistically significant difference from the control treatment ($P < 0.05$)

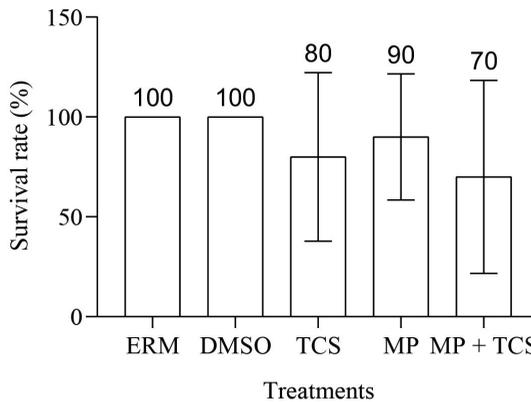


Figure 7: Survival rate of embryo *Oryzias javanicus* were incubated in treatment group

cardiovascular system. The heartbeat is a crucial indicator of life in developing embryos and its absence usually signifies a cessation of vital physiological processes (Cowan *et al.*, 2023).

In this study, embryos exposed to PP+TCS had the lowest survival rate, followed by embryos exposed to TCS. This strengthens the heartbeat data, indicating the influence of the TCS contaminant and effect of PP+TCS. The presence of these contaminants over an extended period can impair the function of vital organs, such as the heart, leading to a weakened heartbeat (bradycardia) in this study. Identifying the absence of a heartbeat is crucial in monitoring embryonic development as it serves as a significant milestone in assessing the embryo's viability. Embryo death may occur due to various factors, including genetic abnormalities, environmental stressors, or insufficient maternal support.

If the heart is unable to pump blood with sufficient strength, the organs will not receive an adequate supply of blood and oxygen to support their functions, leading to death. Meanwhile, embryos that survive until hatching demonstrate resilience to environmental stressors or pressures by employing adaptive survival capabilities, as observed in this study such as delaying the hatching time and having shorter larval size. This phenomenon is known as bet-hedging, an adaptive embryo strategy to enhance the chances of survival in uncertain or changing environments (Mercer, 2021).

PP+TCS may decrease survival rates, as observed in this study, where PP+TCS exposure led to reduced embryo heart rates in the late or pre-hatching stages. This decrease in heart rate could potentially result in mortality due to weakened cardiac function, consequently reducing embryo survival rates.

Conclusions

Heart rate, hatching time, total body length of the newly hatched larvae, and survival rate are the most sensitive biomarkers for detecting the microplastic pollutants polypropylene and

triclosan. The embryos of *O. javanicus* show a synergistic impact when exposed to triclosan adsorbed on polypropylene microplastics. The observed synergistic impact when embryos are exposed to triclosan adsorbed on polypropylene microplastics suggests that the combined presence of these pollutants may have a more pronounced effect than their individual impacts. This emphasises the need for a comprehensive understanding of the interactions between different pollutants in the environment.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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