# Toxicological Effects of Tributyltin in Zebrafish (*Danio rerio*) Embryos

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**ABSTRACT**

Tributyltin (TBT) is known as an endocrine-disrupting chemical abundant in the aquatic environment. In the present study, zebrafish fish embryos were used to observe the chronic toxicity of TBT. Fish embryo toxicity analysis was carried out for different TBT concentrations (100, 50, 25, 12.5, 6.2, and 3.1 ng/L) and fertilized eggs were used to test each concentration effect. Fertilized eggs in 24-well plates (20 eggs in each well) were incubated at 26°C for four days and embryo coagulation, heartbeat of the embryo and mortality lethal endpoints ($LC_{50}$ values) were recorded after 8, 24, 48, and 96 h. The results revealed that 100% coagulations of the embryos occurred at TBT doses of 50 and 100 ng/L. The coagulation significantly increased in a dose-dependent manner and TBT might induce coagulation of zebrafish embryos. Heartbeat changes were significantly decreased ($p<0.05$) in a dose-dependent manner at different TBT doses. $LC_{50}$ values of TBT for zebrafish embryos were 19.9, 11.7, 7.3, and 5.2 ng/L at 8, 24, 48, and 96 h, respectively. The percentage of mortality was higher in embryos for the trace level of TBT, indicating that embryos are more sensitive to TBT toxicity. Hence, TBT is highly toxic and leads to a lethal effect on the zebrafish embryo, resulting in species extinction and declining biodiversity in the aquatic environment.

## 1. INTRODUCTION

Organotin compounds (OTs) are organometallic compounds that have been classified as Persistent Organic Pollutants (POPs) in the environment (Guruge et al., 2008; Ohura et al., 2015). Most OTs come from anthropogenic activities, with industrial production beginning in the 1960s (Sousa et al., 2014). Organotin chemicals are used as effective biocides, polyvinyl chloride stabilizers and industrial catalysts to manufacture silicone and polyurethane foams (Ain et al., 2021; Sousa et al., 2014). Organotins do not decompose thermally and have a low water solubility, allowing them to stay in the aquatic environment for a long time (Cui et al., 2011). Trisubstituted forms of organotin have the highest toxicological activity, and the triorganotins are the most toxic OTs; tributyltin (TBT) is the most toxic form and, considering their reproductive toxicity, OTs are also known as environmental hormones (Guruge et al., 2008; Sekizawa et al., 2003), which disrupt invertebrate and vertebrate reproductive cycles (Langston, 2020). A global ban on a number of OT substances and compounds was imposed in 2008 due to the harmful effects of OTs on living beings and aquatic habitats (IMO, 2001). Tributyltin is still utilized in agriculture and other industrial activities in developing countries with poor environmental monitoring due to its high effectiveness and low cost as antifouling biocides. With the busy maritime and heavy boating and shipping activities in coastal areas all around Sri Lanka and other industrial activities, high TBT usage and contamination in Sri Lanka have been recorded (Bandara et al., 2021). However, there are no records of the effect of TBT contamination to date.

Tributyltin has a high affinity for organic matter (Xiao et al., 2020) and has a half-life of 10-40 years in water and sediments (Guruge et al., 2008; Ohura et al., 2015). This could lead to an increase in their persistence in the environment, which is dependent on a large number of complex chemical structures and physicochemical characteristics (Corsi et al., 2020). As a result, TBT has hydrophobic, lipophilic, and ionic

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properties, which contribute to their toxicity to living organisms. These chemicals rapidly traverse cell membranes and accumulate in the adipose tissues of organisms due to their lipophilicity (Griffin et al., 2020). Their ionic characteristics improve their binding capacity to proteins like α-keratins and glutathione (Lv et al., 2021). Thus, TBT accumulates in living organisms following biomagnification along food chains (Bandara et al., 2021; Fent, 2004; Strand et al., 2003). Many harmful organic compounds accumulate in biota at different concentrations of magnitude and higher than in the ambient environment. These toxicants adversely affect the embryonic and larval stages of biota. This could result in the extinction and decline of affected species in the environment, reducing the diversity of the marine aquatic environment (He et al., 2020; Kim et al., 2018).

Recently, many toxicological studies have used fish and their different life stages as toxicological models due to many endocrine-disrupting chemicals (EDCs) being abundant in the aquatic environment. The results with early life stages of fish predict that about 70% of the long-term toxicity cases are from toxicants such as OTs (Lu et al., 2021). Therefore, the Fish Embryo Toxicity (FET) test would be an auspicious and less time-consuming tool for chemical testing (Santos et al., 2018). Since 2005, the fish embryo toxicity test has been required as part of routine whole effluent testing in Germany, and it has already been standardized at the international level (Lammer et al., 2009). With the majority of research relating to FET results focusing on Lethal Concentration (LC) values, coagulation and lack of heartbeat are the more commonly mentioned endpoints.

Most embryo toxicity tests have been conducted with zebrafish in the laboratories. There are numerous advantages of zebrafish embryo tests such as (1) a mature female zebrafish lays a large number of eggs (50-500) per time (Braunbeck et al., 2005). In the laboratory conditions, different life stages of embryos can easily be produced daily and parallel experimental treatments could be obtained (Scholz et al., 2008). Therefore, large numbers of analysis could be tested at the same time. (2) Due to the small size of embryos, the tests are conducted in 24-well plates. Hence, the tests require a small amount of substances which is particularly important when only limited test solutions are available. (3) The results of the embryo tests can be obtained rapidly (within two or three days) and less time-consuming (4) Sub-lethal endpoints are easy to identify. (5) Fish embryo toxicity tests are more sensitive than the fish cytotoxicity tests and the correlation between the acute fish embryo test and the fish test is perfect (Lungu-Mitea et al., 2020). In this study, zebrafish fish embryos were used to observe the chronic toxicity of TBT by covering the environmentally relevant concentrations of TBT (1, 10, and 100 ng/L) because no previous research in this area had been performed in Sri Lanka.

## 2. METHODOLOGY

### 2.1 Fish and rearing conditions

Wild-type zebrafish; *Danio rerio* were purchased from Angel aquarium, Sri Lanka. Fish were acclimatized for 14 days in glass tanks and the fish tanks were maintained in the laboratory at the Department of Zoology, University of Sri Jayewardenepura. A fourth-generation fish strain was derived from its own stock facilities and used to obtain the embryos.

### 2.2 Egg production of Zebrafish

The day before a test, male and female fish were placed in breeding chambers in 1:1 ratio immediately before the onset of darkness. Artificial plants were used as breeding stimulants and substrates. Mating, spawning and fertilization were observed after 30 min of the onset light in the morning. About 30-60 min after spawning, the spawning dishes were removed. Fertilized and non-fertilized eggs were separated and only fertilized eggs were transferred to a new tank with water. After determining of the overall egg number, viable (fertilized) eggs were kept for hatching. Fertilized eggs were distinguished by their transparency from non-fertilized eggs.

### 2.3 Fish embryo toxicity test (FET) with TBT

Standard TBT chloride and other chemicals (HPLC grade) were purchased from Sigma-Aldrich in Germany.

Fish embryo toxicity analysis was carried out for six different TBT concentrations (half diluted concentrations) of 100, 50, 25, 12.5, 6.2, and 3.1 ng/L (Log concentration 2, 1.7, 1.4, 1.1, 0.8, and 0.5 respectively). According to the method described by Lammer et al. (2009) fish embryo toxicity test was carried out with some modifications in the present study. In brief, embryo tests were initiated after 3 h. Twenty (20 eggs) fertilized eggs were selected for each test concentration and transferred to 24-well plates filled with 2 mL freshly prepared TBT concentrations (test solutions) and negative control (water). Fertilized

eggs were placed in the 24-well-plates by using a sterilized pipette under the dissecting microscope. The 24-well plates were then covered with self-adhesive foil and incubated at 26±1°C for three days. Coagulation, the heartbeat of the embryo and mortality lethal endpoints were recorded using a dissecting microscope after 8, 24, 48, and 96 h. $LC_{50}$ values were determined by graphing mortality percentage versus Log TBT concentrations. Triplicate experiments were performed for the FET test.

### 2.4 Statistical analysis

All assays were carried out in triplicates in three different experiments. All data are represented as the mean±standard deviation. Statistical comparison of the data was carried out using Pearson correlation and regression correlation of Minitab 17 for windows. The correlation value of p less than 0.05 was considered to be a statistically significant linear relationship.

The $LC_{50}$ values were calculated using probit analysis. Statistical analyses were performed using SAS software and linear regression, version 2.17. A significant difference was considered as $p<0.001$ - highly significant.

## 3. RESULTS AND DISCUSSION

In the south Asian region, the lack of advanced detection methods for xenobiotics and their adverse effects are the major challenges for minimizing the trace level of pollutants and their massive impacts on the environment of Sri Lanka (Bandara et al., 2021). Therefore, in the present study, the embryonic effect of TBT was assessed for the first time in Sri Lanka.

### 3.1 Coagulation effect of zebrafish embryo

TBT exposure might cause the coagulation of the embryos. After 48 h, most of the embryos were coagulated and the embryos that did not coagulate showed normal development. A statistically significant increase in embryonic coagulation was observed at different TBT doses of 3.1, 6.2, 12.5, 25, 50, and 100 ng/L compared with the control group ($p<0.05$). The results revealed 100% coagulations of the embryos at TBT doses of 50 and 100 ng/L. Therefore, the coagulation is significantly increasing in a dose-dependent manner (Figure 1) and TBT might induce coagulation and infertility of the zebrafish embryos.

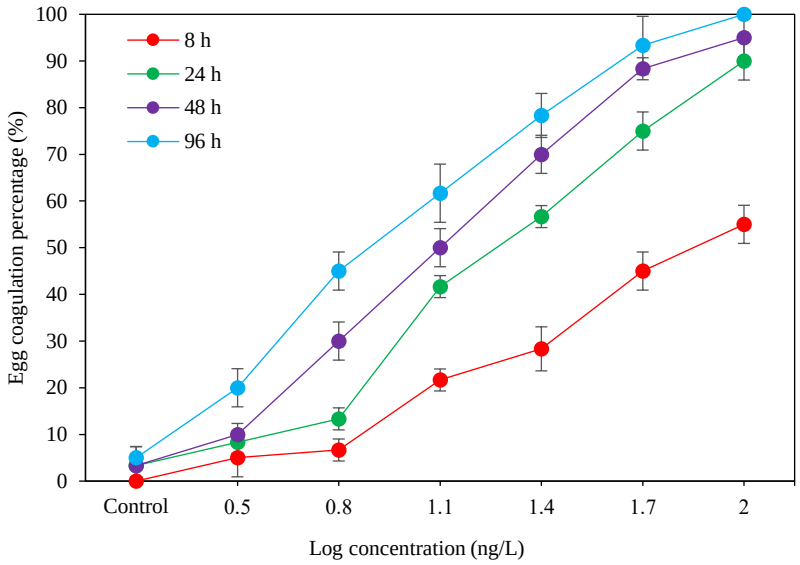


**Figure 1.** Effect of TBT exposure to the embryo coagulation of zebrafish; bpm; beats per minute

Mendis et al. (2018) revealed that coagulation of embryos is a common lethal effect with malathion compounds and it is consistent with the results of the present study. In the current test, the individual eggs were obtained from the same broodstocks and replicated to increase the reliability of the results. Low mortality rates observed in control indicate the experiment's reliability and it was essential to assume there was no inert material effect in the 24 well plates.

### 3.2 Heart toxicity of zebrafish embryo

As shown in Figure 2, heartbeat changes were found in a dose-dependent manner with, according to the results, a statistically significant decrease

occurring at different TBT doses of 3.1, 6.2, 12.5, 25, 50, and 100 ng/L compared with the control group ($p<0.05$). This study demonstrated that TBT exposure caused a significant decrease in heart rate in a dose-dependent manner (Figure 2), and TBT might induce heart toxicity in the zebrafish embryos.

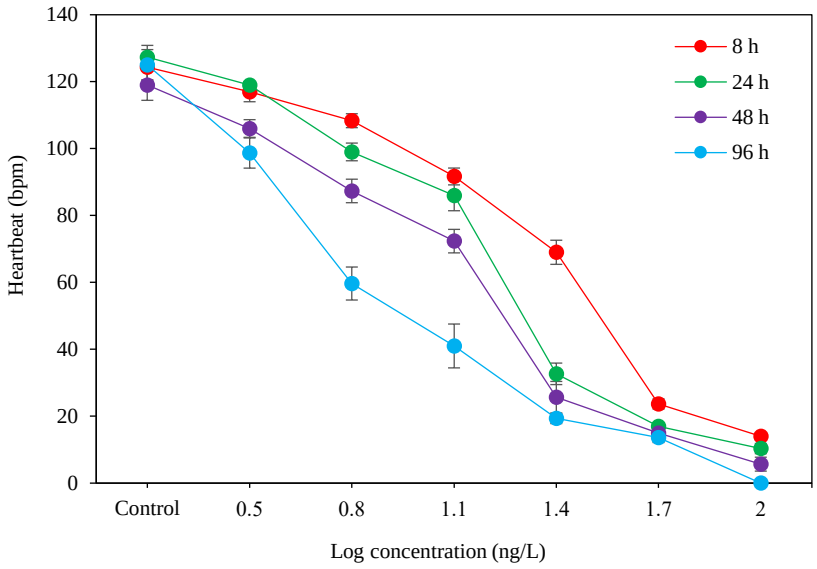


**Figure 2.** Effect of TBT exposure to the embryo heartbeat of Zebrafish; (n=20) bpm; beats per minute

The results revealed that tributyltin is highly toxic and lethal for the zebrafish embryo. After 48 h, most of the embryos were coagulated, and the heartbeat was drastically decreased. Both of these effects were statistically significant and in a dose-dependent manner. Treated embryos exposed to 100 ng/L and 50 ng/L showed total lethality while the control group had no effect. Similarly, Thiti-narongwate et al. (2021) documented that the toxic effect of Zingiber essential oil appeared to occur in a concentration-dependent manner. The lowest dose of oil and control showed no mortality in the zebrafish embryos and a significantly increased mortality rate was in zebrafish embryos exposed to a high dose of oil. There was no viable zebrafish embryos in the groups treated with higher concentrations. The present study was aimed to observe the heart toxicity of zebrafish embryos against different TBT concentrations. The regular heartbeat rate of zebrafish embryos ranges from 120 to 180 per minute (Thitinarongwate et al., 2021). Heartbeat rate was not detected in the embryos at high TBT concentrations due to their early mortality.

### 3.3 Mortality rate and $LC_{50}$ determination

The cumulative mortality of the embryos after TBT exposure is shown in Figure 3. Mortality was recorded at 8, 24, 48, and 96 h in order of increasing concentrations (Log concentration 0.5, 0.8, 1.1, 1.4, 1.7, and 2.0 ng/L). The median lethal concentration ($LC_{50}$) of TBT was calculated by sigmoidal regression using SAS software based on the zebrafish lethality curves (Figure 3). There was a significant ($p<0.0001$) increase in mortality of embryos in response to increasing TBT concentrations (Table 1).

**Table 1.** $LC_{50}$ values of TBT at different time intervals

| Exposure time (h) | $LC_{50}$ (ng/L) | $r^2$ value | p value |
|---|---|---|---|
| 8 | 19.9 | 0.9777 | <0.0001 |
| 24 | 11.7 | 0.9343 | <0.0001 |
| 48 | 7.3 | 0.9731 | <0.0001 |
| 96 | 5.2 | 0.9816 | <0.0001 |

The median lethal concentration ($LC_{50}$) values of TBT for zebrafish embryos were 19.9, 11.7, 7.3, and 5.2 ng/L at 8, 24, 48, and 96 h, respectively. The linear transformation of percentage mortality of embryo and TBT concentration are shown in Figures 3(a), 3(b), 3(c), and 3(d) for different exposure time. Remarkably, the percentage of mortality was higher in embryos for the trace level of TBT, indicating that embryos are more sensitive to TBT toxicity. Exposure to TBT for 8 h or more always caused mortality, increasing with longer exposure times. A continuous exposure until 96 h showed the highest toxicity ($LC_{50}$=5.2 ng/L). During the first 8 h of exposure, the

compound showed less toxicity ($LC_{50}$=19.9 ng/L) compared to those exposed for 96 h post-fertilization ($p<0.0001$) when the embryos were already hatched. This result suggests that the continuous exposure of TBT causes the most toxicity with the lethal endpoint.

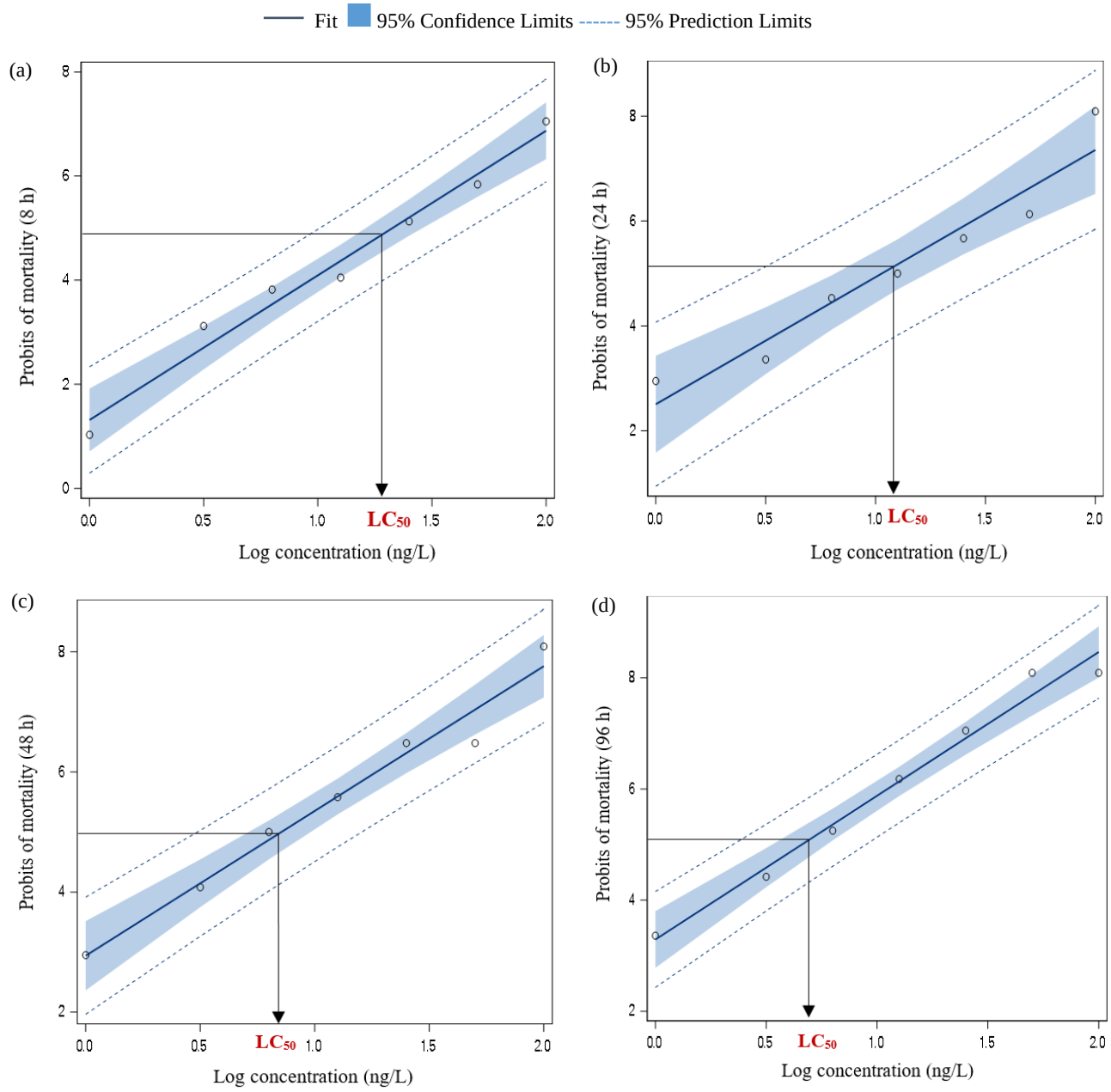


**Figure 3.** The linear transformation and relationship of probit at different concentrations of TBT were used to determine $LC_{50}$ values for zebrafish embryo after (a) 8 h, (b) 24 h, (c) 48 h, and (d) 96 h of exposure to TBT.

The 50% lethal concentrations for each TBT concentration were calculated based on the egg embryos' mortality rate (0-100%). The concentrations were obtained in a geometric series in which each was 50% greater than the next lowest value as recommended by De Oliveira (2018). $LC_{50}$ value was obtained based on cumulative mortality according to the three independent experiments using Regression Probit analysis. The results showed the most of the mortality in 100 and 50 ng/L after 8 h. $LC_{50}$ values were high during the short exposure period and regularly reduced with increased exposure time. The $LC_{50}$ values at 8, 24, 48, and 96 h were obtained as 19.9, 11.7, 7.3, and 5.2 ng/L, respectively. This significant reduction indicates the adverse chronic toxicity of TBT. These results are comparable to the findings of Dimitriou et al. (2003), which revealed that fish (Seabream fish) fertilized eggs were quite

sensitive to both TBT and triphenyltin while $LC_{50}$ for TBTCl and triphenyltin were 28.3 and 34.2 ng/L, respectively at 24 h. Moreover, much lower $LC_{50}$ values at 96 h (<5 ng/L) have been reported for the embryo of two freshwater fish species, *Brevoortia tyrannus* and *Menidia beryllina* (Bushong et al., 1988). Therefore, TBT is responsible for the time-related toxicity of the embryos (Dimitriou et al., 2003). The concentrations of TBT compounds in studied water and sediments often exceed these tested levels. Consequently, fish embryos at the contaminated water bodies are at high risk since they float on the water surface or adhere to the benthic sediments. Even at low TBT concentrations, extremely low $LC_{50}$ values could be estimated in these extreme cases of TBT pollution. It may cause direct mortality in aquatic animals during their early developmental stages. Bushong et al. (1988) observed that survival of euryhaline sheepshead minnow (*Cyprinodon variegatus*) was reduced to 59% compared to control when exposed to TBT from hatching to maturation and reproduction at 0.66 mg/L by day 145 and 100% at 5.4 mg/L by day 7, while survival and growth of the following generation were only unaffected at concentrations lower or equal to 1.3 mg/L. Furthermore, Pinkney et al. (1990) found significantly reduced larval survival in the first generation of euryhaline striped bass (*Morone saxatilis*) at TBT concentrations above 0.77 mg/L.

The present study identified that TBT might be a reason for reproductive impairments in zebrafish embryo. The findings may reveal that TBT pollution in water bodies in Sri Lanka might be an additional factor responsible for reducing natural populations in the environment by affecting the embryos and the larvae of animals. Human health implications from contaminated fish consumption should also be considered. Based on all this evidence, there is a need to develop safety and precautions regarding TBT usage. There is a necessity to be concerned that TBT might be entering into the human food chain through marine aquaculture of mollusks and fish despite the controls implemented in 1992. This study suggests that an extended monitoring program is required to check whether the 1992 legislation controlling the use of TBT is being enforced and whether it will be effective in the long term.

## 4. CONCLUSION

In this study, the chronic toxicity of TBT was assessed by using zebrafish embryos as a promising model to investigate the toxicity of hazardous chemicals. Coagulation, the heartbeat of the embryo and mortality lethal endpoints were recorded as the primary toxicological assays in the world. The results show that the coagulation and lack of heartbeat significantly increased with increasing TBT concentrations and the exposure time in a dose-dependent manner. $LC_{50}$ values of TBT for zebrafish embryos were drastically decreased with increasing exposure time. The lethality was higher in embryos for the trace level of TBT, indicating the adverse chronic toxicity of TBT for the fish embryos. These adverse effects on fish embryos could easily threaten the ecosystem and biodiversity in Sri Lanka if tributyltin use is not strictly regulated.

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## CONFLICTS OF INTEREST

The authors declare that they have no competing of interest as all were worked together and the manuscript has not been submitted simultaneously for publication elsewhere.

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