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# EFFECTS OF TEMPERATURE AND METALS (ZINC AND CADMIUM) IN EMBRYONIC STAGE OF *Anabas testudineus*: AN ALTERNATIVE FRESHWATER FISH EMBRYO TEST IN THE SOUTHEAST ASIA REGION

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## ABSTRACT

The fish embryo acute toxicity test (FET) was proposed as a promising alternative to the general practice of fish acute toxicity test (FAT). To date, the available information on freshwater fish embryos in Southeast Asia is limited. Hence, this study aims to present preliminary data on single and combined exposure effects of temperature and metals (zinc and cadmium) on the embryonic development of *Anabas testudineus*. Over 80% of the embryos died after 10 h post-fertilization (hpf) in 1.59, 7.02, and 13.0 mg/L cadmium treatments, whereas the same effect was only observed in 49.6 and 146 mg/L of zinc treatments as early as 8 hpf. The optimum temperature for embryos ranged from 27 to 34 °C, with a survival rate greater than 90% was recorded. The combined test of zinc + cadmium showed the additive effect with approximately 80% mortality at 12 hpf, while the combination of temperature + cadmium had increased the mortality rate up to 100% at 16 hpf. The constant temperature even at optimum rearing value was found to increase cadmium uptake in the embryos and magnified the concentration higher than in water. Therefore, this study suggests climbing perch (*Anabas testudineus*) could be an alternative FET test model in this region.

**Key words:** Abnormality, bioconcentration factor, climbing perch, $LC_{50}$, toxicity test

## INTRODUCTION

Nowadays, the current trend using fish embryo acute toxicity (FET) test has been proven as a promising alternative to fish acute toxicity (FAT) test (Klüver *et al*., 2015). The experimental model using FET test organisms was approved by the Working Group of the National Coordinators (WNT) of the Organisation for Economic Co-operation and Development (OECD) Test Guideline Program and published as OECD test guideline no. 236 on 2013 (TG236, 2013). Moreover, the embryotoxicity model has been verified as a valuable tool in assessing chemical and effluent toxicity effects in the early life stage of fish (Schulte & Nagel, 1994; Braunbeck *et al*., 2005; Lammer *et al*., 2009; Embry *et al*., 2010; Hermsen *et al*., 2011) due to its high sensitivity, fast, simple and inexpensive (Chahardehi *et al*., 2020). The common FAT test approach in Germany has been replaced with the FET test since 2003 using zebrafish (*Danio rerio*) embryos (Din, 2001; International Organization for Standardization, 2007).

The zebrafish (*Danio rerio*) embryo commonly used as a freshwater embryotoxicity model in Southeast Asia which were exposed to a vast number of toxicants (Hallare *et al*., 2009; Alafiatayo *et al*., 2019; Omar *et al*., 2020; Ramli *et al*., 2020; Damodaran *et al*., 2021; Kitipaspallop *et al*., 2021; Thitinarongwate *et al*., 2021). Besides zebrafish, walking catfish (*Clarias macrocephalus*) and climbing perch (*Anabas testudineus,* Malaysia) (Nurulnadia *et al*., 2020; Wankanapol & Vera Cruz, 2020) embryos were also used in Southeast Asia. Development research on zebrafish has indeed become an important part of the biological field (Kinth *et al*., 2013), but this species surely has its drawback especially to a beginner as well as in an underdeveloped research facility. Zebrafish stock should be kept in a circulating system that continuously filters and aerates the system water (Avdesh *et al*., 2012), hence requiring a proper set-up of rearing conditions and electricity supply. In the case of *A. testudineus*, a simple 10 L tank can accommodate approximately 2 individuals of fish in the range of 50 – 100 g of fish weight, and neither electric nor aeration is required. The

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endpoint of hatching of zebrafish generally lasts up to 72 h compared to only 20 h in *A. testudineus* in the present study (Nurulnadia *et al*., 2020). Therefore, the present study was preliminary conducted to establish climbing perch (*Anabas testudineus*) embryo as an alternative FET test organism to common approach, zebrafish (*Danio rerio*), specifically in Southeast Asia region, the native region of this species.

Climbing perch or *Anabas testudineus* is widely distributed in the Southeast Asia region including Malaysia, Thailand, Vietnam, Philippines, and Indonesia (Al-Rasheed *et al*., 2018). It is a freshwater fish that can adapt and proliferate in ponds, lakes, swamps, rivers, and paddy fields (Nurulnadia *et al*., 2020). Therefore, the broodstock of this species can be easily caught alive from the field or bought from an aquarium shop. Besides, the hatching time of this species was recorded approximately 19.5 to 20 h post-fertilization (hpf) (Nurulnadia *et al*., 2020), requires less observation period compared to other FET test species (zebrafish 72 h in Avdesh *et al*., 2012; Japanese medaka 9 days in Kawano *et al*., 2017). The developmental stage of less than 24 h with good endpoint observations (etc. hatching rate, mean hatching h, unhatched, mortality, and abnormality) was observed in the same study. A shorter embryonic development stage could be an advantage to establish a simple and fast embryotoxicity model for the FET test (Chahardehi *et al*., 2020). Moreover, extensive rear-out facilities are not required for the broodstocks as they can be kept in a static water aquarium (Parvin *et al*., 2010). A good embryotoxicity model should fulfill criteria such as short-developmental stages, easy culturing, transparent fish eggs, and higher fecundity (Volckaert *et al*., 1994). These criteria were observed in *A. testudineus* embryo (e.g Abdul Ghani, 2016; Nurulnadia *et al*., 2020), indicating a good alternative for FET.

Malaysian river waters were reported to contain a significant amount of cadmium and zinc (Rosli *et al*., 2018; Razak *et al*., 2021; Nurulnadia *et al*., 2021). This becoming a concern as previous studies showed sublethal effects in embryonic development of fish that were exposed to cadmium (Ismail & Yusof, 2011; Zhang *et al*., 2012; Witeska *et al*., 2014) and zinc (Huang *et al*., 2010; Küçükoğlu *et al*., 2013). The sensitivity of embryos towards toxicants could be triggered by confounding influences such as temperature (Hallare *et al*., 2005). Therefore, this study aims to conduct toxicity tests on single and combined treatments of temperature and two metals (cadmium and zinc) using *A. testudineus* as an alternative embryotoxicity model. The study is one of the initial efforts in conducting and compiling preliminary toxicity data using *A. testudineus* embryos as regional FET for Southeast Asia.

## MATERIALS AND METHODS

### Cultural and spawning

The broodstocks of *A. testudineus* were purchased from a local aquarium store in Kuala Terengganu, Malaysia. The fish stocks were acclimatized in dechlorinated tap water at room temperature (27 – 31 °C) for almost two years in Universiti Malaysia Terengganu to observe the feeding pattern, swimming behavior as well as physical health. Only healthy broodstock with normal feeding and behavior was selected for the production of eggs. The bodyweight of male and female fish was recorded between 22.5 – 26.5 g and 48.9 – 59.3 g, respectively. They were kept in a 50 L aquarium tank and fed twice daily based on 5% of their body weight using a commercial diet pellet (Aqua Collection Growth, Malaysia). The water parameters such as pH (5.66 ± 0.27), temperature (26.69 ± 1.80 ºC), and dissolved oxygen (5.29 ± 1.74 mg/L) were measured and maintained periodically.

The hormone injection procedures were adopted from Nurulnadia *et al*. (2020). One female and two males with the healthy condition was injected with 0.5 mL/kg (female) and 0.25 mL/kg (male) Ovaprim hormone (Syndel, Canada) and kept in a 10 L tank for spawning. Fertilized eggs were produced naturally between 10 and 12 h after injection, and selection of fertilized eggs was conducted right away under the compound microscope. The embryos were only subjected to the test if the fertilization rate was higher than 80% following OECD test guidelines (TG236, 2013). This percentage was determined by measuring the number of fertilized eggs over a total number of eggs produced in 100 mL of water. Fertilized embryos were washed thee times with water before being transferred to a petri dish containing rearing water (dechlorinated tap water).

### Preparation of experimental solutions

Anhydrous zinc chloride ($ZnCl_2$; MW: 136.29, Sigma Aldrich, Malaysia) and cadmium chloride ($CdCl_2$; MW: 183.32, Sigma Aldrich, Malaysia) were used to prepare a 400 mg/L stock solution, respectively. The nominal concentration of zinc and cadmium in a single test ranged from 2 to 150 and 0.5 to 15 mg/L, respectively. The nominal concentration of combination zinc and cadmium ranged from 2 – 50 mg/L with constant 2 mg/L cadmium. The solution was prepared using dechlorinated tap water. The calculated volume of target zinc or/and cadmium concentration was added into dechlorinated tap water and ultrasonicated for 15 min before exposure to embryos.

### Range finding test of cadmium and zinc

The range-finding tests on cadmium and zinc were experimented with before conducting the final toxicity tests. To our knowledge, no published

work reported concentrations of zinc and cadmium on *A. testudineus* embryos, hence concentrations were referred to as zebrafish (*Danio rerio*) embryos (Hallare *et al*., 2005; Ansari & Ansari, 2015). The nominal concentration in the single test mentioned above aims to calculate median lethal concentration ($LC_{50}$) at 24 h as well as computing the low-observed effect concentration (LOEC) and no-observed effect concentration (NOEC) values. The 24 h $LC_{50}$, LOEC and NOEC were determined using logarithm graph in Microsoft Excel 2013 by employing the equation y = a ln(x) + b; which y = mortality, a = logarithm coefficient, x = concentration and b = interception. $LC_{50}$ was determined by replacing the y-value to fifty (50), whereas LOEC was picked based on the lowest concentration that causes embryo mortality and NOEC was computed by replacing the y-value to zero (0).

**Single and combined toxicity test of temperature, zinc, and cadmium**

The experimental framework for both single and combined toxicity tests of temperature, zinc, and cadmium can be summarized as shown in Figure 1. Acute toxicity test using fish embryo was conducted following OECD guideline no. 236 (TG236, 2013). The experiment was conducted in an incubator oven (Binder, Germany) while, control treatment was placed at room temperature (26.7 – 29.5 °C). Before exposure, dechlorinated tap water in a 50 mL beaker was placed in an incubator to obtain target temperatures (Table 1) for 24 h. One treatment group consists of 30 embryos with 3 replications each (Figure 1). The observation of embryos was done at 2, 4, 8, 12, 16, 20, and 24 h of hpf. During the observation period, the embryos were picked up and observed using a compound microscope (CM-46; Leica, Germany). The embryos assigned to temperature treatment were observed in less than 10 min and returned to the incubator again until the end of the experimental period. A similar experimental set-up was conducted to expose *A. testudineus* to zinc and cadmium at room temperature (26.7 – 29.5 °C). The combined exposure treatments were conducted at optimum temperature and median lethal concentration (24 h $LC_{50}$) values (Table 1). The measured concentrations for both single and combined exposure treatments were summarized as shown in Table 1.

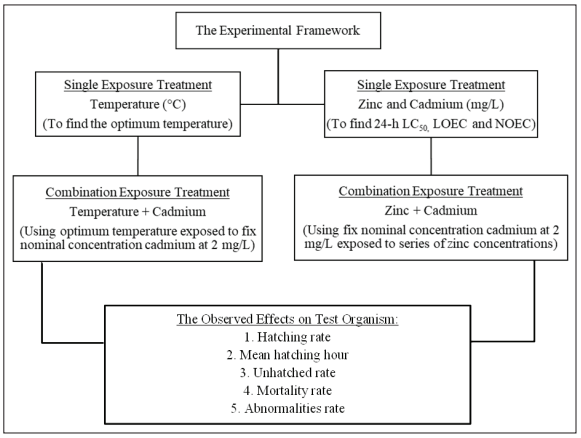



**Fig.1.** The experimental framework on single and combined exposure treatments of temperature, zinc, and cadmium.

**Table 1.** The measured temperature (°C) and concentrations of zinc and cadmium (mg/L) in water

| Single exposure treatment | | | | |
|---|---|---|---|---|
| Temperature (°C) | | Cadmium (mg/L) | | Zinc (mg/L) |
| 22 | | 0.13 | | 2.63 |
| 27 | | 0.94 | | 4.20 |
| 34 | | 1.59 | | 13.0 |
| 40 | | 7.02 | | 49.6 |
| 45 | | 13.0 | | 146 |
| Combined exposure treatment | | | | |
| Temperature (°C) | | Cadmium (mg/L) | | Zinc (mg/L) |
| 27 | + | 1.40 | | na |
| 34 | + | 1.73 | | na |
| | | 1.85 | + | 2.98 |
| Room temperature | | 2.05 | + | 21.60 |
| (26.7 – 29.5) | | 1.90 | + | 30.60 |
| | | 1.79 | + | 34.80 |

[a]The combined exposure treatments: temperature + cadmium and cadmium + zinc
Temperature value fluctuated by ±1
na – not available

**Morphological abnormalities of embryos**

The abnormalities of *A. testudineus* embryos were observed similarly following Nurulnadia *et al*. (2020). In the present study, morphological abnormalities were observed in each embryo (*n*=30) for treatments groups (temperature + cadmium and cadmium + zinc) in every hpf. The abnormality of the embryo was compared to the control treatment and the pictures were taken as evidence. The morphological abnormalities observed included ruptured chorion, shinking of yolk, tail malformation, and coagulation of embryos (Table 4).

**Zinc and cadmium analysis in the water and embryos**

The analysis for cadmium and zinc in water and embryos were performed similarly following the published method by US EPA (1997) and Agusa *et al*. (2005), respectively. Water samples were filtered using Whatman GF/C glass microfiber filter paper (Merck, United Kingdom) and preserved with 10% Suprapur nitric acid (pH < 2). Before measurement, the samples were stored at 4 °C and these procedures were following the published method by US EPA (1997). The embryos from the same treatment group were pooled in a 1.5 mL microcentrifuge tube and the wet weight was recorded. The extraction of cadmium in fish embryos was done following Agusa *et al*. (2005). The cadmium was extracted from the pooled embryos (*n*=30) using 1.5 mL of nitric acid in a Teflon beaker. The metals were extracted from the homogenized pooled embryos (including all developmental stages) from all treatment groups (*n*=30 x 3 replicates) to achieve a minimal 50 mg weight of sample per analysis (Nurulnadia *et al*., 2021). The digestion process was conducted in the heating oven with a temperature of 100 °C for 8 h. The zinc and cadmium concentration in both water and embryos samples were determined by inductively-coupled plasma mass spectrometry, ICP-MS (Perkin Elmer, ELAN 9000, United States).

For quality assurance purposes, SLRS-6: (River Water Certified Reference Material for Trace Metals and other Constituents, Canada), DOLT-4 (Dogfish Liver, Canada), and blank were subjected to the same extraction procedures. Mean percent recovery for cadmium in water and fish was recorded at 72 and 75%, with the detection limit of 0.010 mg/L and 0.001 mg/kg, respectively. The analysis was conducted in triplicate (*n*=3) and the error of all analysis were within ± 25%. The cadmium concentration in the control treatment (both water and embryo) was recorded below the detection limit.

**Computation of hatched, unhatched, abnormality and mortality percentage**

The observed effects include hatched, unhatched, mortality, and abnormality rate between exposure treatments were analyzed using Microsoft Excel 2019. The correlation between concentrations (cadmium and zinc) with mortality rate was computed using IBM SPSS Statistic 25 software. The calculation of hatched, unhatched, and mortality and abnormality rate were calculated as the following:

Hatched rate (%) = number of hatched embryos/ total number of embryos x 100
Unhatched rate (%) = number of unhatched embryos/total number of embryos x 100
Mortality rate (%) = number of dead embryos/total number of embryos x 100
Abnormality rate (%) = number of abnormal embryos/total number of embryos x 100

**RESULTS AND DISCUSSION**

Based on the characterization, all embryos in control treatment for both single and combined tests showed a 100% survival rate with the hatching period ranging from 16 to 18 hpf. The temperature, dissolved oxygen, and pH values were recorded in the range of 26.4 – 27.7 °C, 5.0 – 6.3 mg/L, and 5.0 – 6.2, respectively, during all the exposure series. The tests were conducted in optimum conditions as indicated by the number of survived and hatched embryos in the control treatment.

**Endpoint values of zinc and cadmium**

Table 2 shows the median lethal concentration ($LC_{50}$), lowest observed effect concentration (LOEC), and no observed effect concentration (NOEC) values of each zinc and cadmium test in several types of embryos. In terms of sensitivity, *A. testudineus* embryos were not as sensitive as rainbow trout (*Salmo gairdneri*) (Van Leeuwen *et al*., 1990) towards zinc and fathead minnows (*Pimephales promelas*) (Birge *et al*., 1985) to cadmium. However, the $LC_{50}$, LOEC, and NOEC values of both zinc and cadmium in *A. testudineus* embryos in the present study are comparable to the African catfish (*Clarias gariepinus* in Nguyen & Janssen, 2001) (see Table 2). The only difference was the exposure period. *Anabas testudineus* embryos in this study were exposed only in 24 h compared to 120 h in African catfish embryos. If the period was to be extended, *Anabas testudineus* embryos might show lower $LC_{50}$, LOEC, and NOEC values. Therefore, *A. testudineus* can be as sensitive as the other types of FET available to date. This study could be used as preliminary data as no report on cadmium and/or zinc testing was previously reported on the same species of *A. testudineus*.

**Single and combined effects on *Anabas testudineus* embryos**

The measured values of zinc, cadmium, and temperatures for both single and combined toxicity tests are as shown in Table 3. A similar trend was

**Table 2.** Endpoints of zinc and cadmium tested on embryos at different exposure periods

| Test embryos (Species) | Period (h) | Zn (mg/L) | | | Cd (mg/L) | | | Location | References |
|---|---|---|---|---|---|---|---|---|---|
| | | $LC_{50}$ | LOEC | NOEC | $LC_{50}$ | LOEC | NOEC | | |
| Fathead minnow (*Pimephales promelas*) | 192 | na | na | na | 0.04 | 0.08 | 0.01 | Kentucky USA | Birge *et al*., 1985 |
| Zebrafish (*Brachydanio rerio*) | 168 | 10.0 | 3.20 | na | na | na | na | Leidschendam, Netherland | Van Leeuwen *et al*., 1990 |
| Rainbow trout (*Salmo gairdneri*) | 1440 | 0.52 | 1.00 | na | na | na | na | | |
| Gastropod (*Physa acuta*) | 24 | na | na | na | 1.27 | na | na | Kowloon, Hong Kong | Cheung & Lam, 1998 |
| | 48 | na | na | na | 0.85 | 0.50 | 0.32 | | |
| African catfish (*Clarias gariepinus*) | 120 | 18.7 | 2.30 | 2.30 | 0.67 | 0.50 | 0.15 | Ghent, Belgium | Nguyen & Janssen, 2001 |
| Zebrafish (*Danio rerio*) | 288 | 2.10 | 1.40 | 0.70 | 0.10 | 0.15 | 0.05 | | |
| Zebrafish (*Danio rerio*) | 24 | na | | na | 24.1 | na | na | Tübingen, Germany | Hallare *et al*., 2005 |
| | 48 | na | | na | 30.1 | na | na | | |
| Red sea bream (*Pagrus major*) | 24 | na | | na | 9.80 | na | na | Qingdao, China | Cao *et al*., 2009 |
| | 48 | na | | na | 6.60 | na | na | | |
| Climbing perch (*Anabas testudineus*) | 24 | 14.8 | 2.63 | 2.05 | 0.67 | 0.13 | 0.06 | Terengganu, Malaysia | Present study |

$LC_{50}$ - Lethal concentration; LOEC – Lowest observation effect concentration; NOEC – No observed effect concentration; na – not available

observed in the single exposure of zinc and cadmium, where the hatching rate was decreased and the mortality rate of embryos was increased in parallel with the measured concentrations. The results showed positive correlations between mortality rate with temperature ($r$=0.870), zinc ($r$=0.934) and cadmium ($r$=0.872). This implies that higher temperatures and concentrations of zinc and cadmium caused a higher mortality rate of the test organisms. Over 80% of the embryos were killed at 8 hpf in the 49.6 and 146 mg/L of zinc which was similarly achieved at 10 hpf in the 1.59, 7.02, and 13.0 mg/L of cadmium. Based on the lethal concentration values ($LC_{50}$), *A. testudineus* embryos showed higher tolerance to zinc (14.8 mg/L) than cadmium (0.67 mg/L) due to the importance of this element as an essential nutrient in living organisms (Yusoff *et al*., 2018). Regarding other studies, the same level of tolerance was only shown in zebrafish and African catfish embryos (Nguyen & Janssen, 2001).

All embryos at the temperature of 40 and 45°C died as early as 8 hpf indicating the lethal effect of extreme heat to fish embryos. Schirone and Gross (1968) discovered that higher temperatures (35 °C) inhibited the embryo development of zebrafish (*Brachydanio rerio*) which resulted in a higher mortality rate. In the present study, the climbing perch (*A. testudineus*) embryos survived at 22 °C thoughout the exposure period with 6.7% mortality however, their growth stopped within 8 to 12 hpf (ten to eighteen somites developmental stage). Similar to the previous studies, the zebrafish embryos maintained at 22 °C were incapable to be developed past the thirty-two cell stage (Schirone & Gross, 1968). The off-limit temperature caused an imbalance in metabolic rates, resulted in the production of abnormal embryos, and/or accelerated or delayed the embryonic development of the fish (Repolho *et al*., 2014; Uriarte *et al*., 2015; Caamal-Monsreal *et al*., 2016; Gamain *et al*., 2017). Most of the embryos survived in the temperature range from 27 to 34 °C, with a mortality rate of less than 6.7%. This study suggests that the optimum temperature for the growth of *A. testudineus* is within 27 to 34 °C.

The combined test of zinc + cadmium demonstrated the lethality effect of additives, with approximately 80% of embryos were killed at 12 hpf in all treatments. The toxicity of a chemical could enhance the positive synergistic and interaction effects with the presence of another toxicant (Fernández & Beiras, 2001). Previous studies showed that larvae of fathead minnow and freshwater shimp were highly affected by the combination of cadmium and zinc exerted during the exposure treatments (Eaton, 1973; Thorp & Lake, 1974). These metals have acted synergistically and dependently interacted to some degree which led to severe detrimental effects on the test organisms. This suggests that the combination of zinc and cadmium pose additive effects to the various life stage of organisms. A total of 13.3% and 3.3% of embryos in the two highest treatment of combined zinc and cadmium (30.60 mg/L zinc + 1.90 mg/L cadmium and 34.80 mg/L zinc + 1.79 mg/L cadmium) were unhatched. The embryos however survived with abnormal development (Table 4). The higher concentration of zinc in these two treatments signifies the outweigh effect of zinc over cadmium on the embryos. Based on previous studies, the effects of zinc on fathead minnow (Brungs, 1969), zebrafish (Dave *et al*., 1987), flounder (Yulin *et al*., 1990), Australian crimson spotted rainbowfish (Williams & Holdway, 2000), and red sea bream (Huang *et al*., 2010) embryos showed a delay in the hatching time due to the interruption of hatching enzymes activities which prolonged the developmental period. Presumably, this could be the reason behind the unhatched embryos of climbing perch in the present study.

The combination of temperature and cadmium had killed 100% of embryos in 27 °C + 1.40 mg/L and 34 °C + 1.37 mg/L treatments at 20 and 16 hpf, respectively. The existence of cadmium had shunk the survival rate of embryos even at the optimum temperature values. Conversely, Hallare *et al*. (2005) reported that the zebrafish embryos survived in high temperature (33 °C) but were reported dead in low temperature (21 °C) during the combined exposure treatment with 10 mg/L of cadmium. This suggests that zebrafish embryos are more tolerant to cadmium with 24 h $LC_{50}$ of 24.1 mg/L (Table 2) compared to climbing perch in the present study. The discovery suggested that the mixture effect of temperature and cadmium on fish embryos might be species-specific. Thus, *A. testudineus* embryos are seemed to be vulnerable to the combination of the physical parameter (temperature) and toxicants (zinc and cadmium), indicating such additives could amplify the detrimental effects on the survival of this species in an ecosystem. These factors require tedious preparation and consideration to be conducted in the field, but the results can be achieved straightforwardly though laboratory trials. Therefore, the experimental approach is undoubtedly a relevant testing method of parameters or chemical toxicants to date (Zhang *et al*., 2012).

**The abnormalities of embryos**

The abnormalities of embryos in the combined test were recorded in percentage values. Among the abnormalities, four criteria were tabulated and presented in Table 4. The most common abnormalities observed were the rupturing of the chorion (the outer cell of an embryo) in a combined test of zinc + cadmium, specifically in zinc concentration greater than 30 mg/L. The condition was commonly observed in 8 hpf and 12 hpf (Table 5). Deformation of chorion was the most proclaimed effect following the exposure to cadmium and zinc (Cheung & Lam, 1998; Huang *et al*., 2010). During the early life stage, the chorion act as a barrier between growing embryos and the toxicants

**Table 3.** Single and combined effects of zinc, cadmium, and temperature on climbing perch (*Anabas testudineus*) embryos

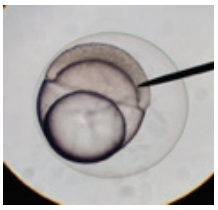
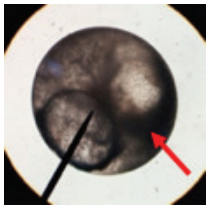
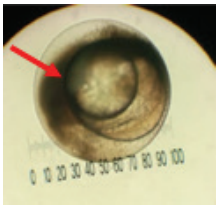
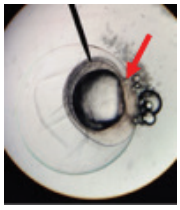
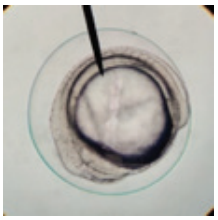
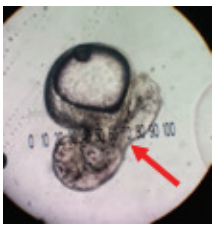
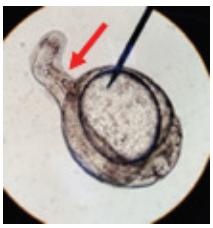
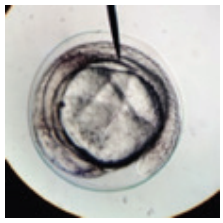
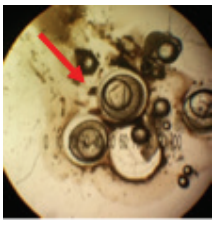
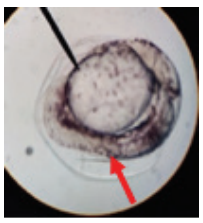
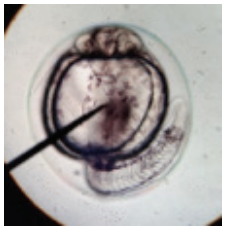
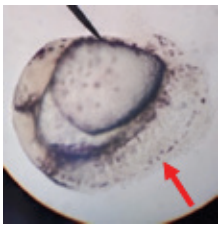
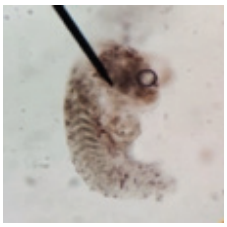
| Single test | Hatched rate (%)[a] | Mean Hatching (hpf)[b] | Unhatched rate (%)[c] | Mortality rate (%)[d] |
|---|---|---|---|---|
| Zinc (mg/L) | | | | |
| Control[e] | 100 | 16.0 | 0 | 0 |
| 2.63 | 76.7 | 16.0 | 20.0 | 03.3 |
| 4.20 | 56.7 | 16.0 | 20.0 | 23.3 |
| 13.0 | 50.0 | 16.5 ± 1.4 | 6.7 | 43.3 |
| 49.6 | 46.7 | 21.4 ± 2.0 | 3.3 | 50.0 |
| 146 | 0 | 0 | 0 | 100 |
| Cadmium (mg/L) | | | | |
| Control[e] | 100 | 16.0 | 0 | 0 |
| 0.13 | 93.3 | 16.0 | 0 | 6.7 |
| 0.94 | 50.0 | 16.0 | 0 | 50.0 |
| 1.59 | 3.3 | 16 | 0 | 96.7 |
| 7.02 | 0 | 0 | 0 | 100 |
| 13.0 | 0 | 0 | 0 | 100 |
| Temperature (˚C) | | | | |
| Control[e] | 100 | 18.0 ± 5.2 | 0 | 0 |
| 22 | 0 | 0 | 93.3 | 6.7 |
| 27 | 96.7 | 22.0 ± 2.8 | 3.3 | 0 |
| 34 | 93.3 | 18.0 ± 5.2 | 0 | 6.7 |
| 40 | 0 | 0 | 0 | 100 |
| 45 | 0 | 0 | 0 | 100 |
| Combined test | | | | |
| Zinc + Cadmium (mg/L) | | | | |
| Control | 100 | 16.0 | 0 | 0 |
| 2.98 + 1.85 | 0 | 0 | 0 | 100 |
| 21.60 + 2.05 | 0 | 0 | 0 | 100 |
| 30.60 + 1.90 | 0 | 0 | 13.3 | 86.7 |
| 34.80 + 1.79 | 0 | 0 | 3.30 | 96.7 |
| Temperature + Cadmium (mg/L) | | | | |
| Control[e] | 100 | 18.0 ± 5.2 | 0 | 0 |
| 27°C + 1.40 | 0 | 0 | 0 | 100 |
| 34°C + 1.73 | 0 | 0 | 0 | 100 |

[a] 100 x (number of hatched embryos/total number of embryos)
[b] Mean ± standard deviation hatching h in h post-fertilization (hpf) (n = 30)
[c] 100 x (number of unhatched embryos/total number of embryos)
[d] 100 x (number of dead embryos/total number of embryos)
[e] Room temperature (26.7 – 29.5 °C)

**Table 4.** Percentage abnormalities (number of abnormal embryo/total embryo) in the combined treatments on Climbing perch (*Anabas testudineus*) embryos

| Treatment | Ruptured chorion | Shinking of yolk | Tail malformation | Coagulation of embryos | Total abnormalities |
|---|---|---|---|---|---|
| Zinc + Cadmium (mg/L) | | | | | |
| 2.98 + 1.85 | 3.33 (1/30) | 3.33 (1/30) | 6.67 (2/30) | 6.67 (2/30) | 20.00 |
| 21.6 + 2.05 | 6.67 (2/30) | 3.33 (1/30) | 3.33 (1/30) | 10.00 (3/30) | 23.33 |
| 30.6 + 1.90 | 16.67 (5/30) | 6.67 (2/30) | 10.00 (3/30) | 10.00 (3/30) | 43.33 |
| 34.8 + 1.79 | 20.00 (6/30) | 10.00 (3/30) | 13.33 (4/30) | 13.33 (4/30) | 56.67 |
| Temperature (°C) + Cadmium (mg/L) | | | | | |
| 27.00 + 1.40 | 16.67 (5/30) | 3.33 (1/30) | 30.00 (9/30) | 13.33 (4/30) | 63.33 |
| 34.00 + 1.73 | 13.33 (4/30) | 3.33 (1/30) | 30.00 (9/30) | 16.67 (5/30) | 63.33 |

**Table 5.** Comparison of fish embryos in 2 – 16 h post fertilization between control (a) and abnormal embryos in single (b, c) and combined treatments (d, e, f, g, h, i, j, k, l)

| Treatments | (a) Control | (b) 45 °C | (c) Cd 7.02 mg/L | (d) Cd 2.05 + Zn 21.60 mg/L |
|---|---|---|---|---|
| 2 hpf |  |  |  |  |
| Abnormality | None | Coagulated embryos | Coagulated embryos | Ruptured chorion |
| Treatments | (a) Control | (e) Cd 1.90 + Zn 30.60 mg/L | (f) Cd 1.79 + Zn 34.80 mg/L | (g) Cd 1.79 + Zn 34.8 mg/L |
| 8 hpf |  |  |  |  |
| Abnormality | None | Blastodermal lesion | Ruptured chorion | Tail malformation |
| Treatments | (a) Control | (h) Cd 1.90 + Zn 30.6 mg/L | (i) Cd 1.79 + Zn 34.8 mg/L | (j) 27 °C + Cd 1.40 mg/L |
| 12 hpf |  |  |  |  |
| Abnormality | None | Coagulated embryos | Ruptured chorion | Tail malformation |
| Treatments | (a) Control | (k) 34 °C + Cd 1.73 mg/L | (l) 34 °C + Cd 1.73 mg/L | |
| 16 hpf |  |  |  | |
| Abnormality | None | Shinking of yolk | Dead | |

hpf – an h of post-fertilization;
Zn – Zinc;
Cd – Cadmium
The abnormalities were pointed using a red arrow.

(Pavlaki *et al*., 2016), and becomes permeable as it is near to hatch. Therefore, chorions are prone to be deformed and ruptured at a later h in this study. In the combined test of temperature and cadmium, tail malformation was the most observed effect at 12 and 16 hpf even after or before hatching, respectively. Tail malformation such as lack or hooked tail (Cao *et al*., 2009) and curled tail (Huang *et al*., 2010) were reported in *Oreochomis mossambicus* and *Danio rerio* embryos following their exposure to cadmium and zinc. The waterborne cadmium might cause $Ca^{2+}$-ATPase activity of myosin and myotome to be reduced to form a normal musculoskeletal system development (Cao *et al*., 2009). Insufficient $Ca^{2+}$-uptake in the embryos could result in tail malformation.

**Measured concentration and bioconcentration factor (BCF) in the embryos**

Bioconcentration factor (BCF) values of zinc and cadmium were calculated for *A. testudineus* embryos and shown in Table 6. The concentration of zinc and cadmium in control water and embryos were below the detection limit. Thus, embryos in this combined test seemed to bioconcentrate both metals from water. BCF values in a single zinc and cadmium test were below 1, suggesting insignificant bioconcentration (Nurulnadia *et al*., 2013; Yusoff *et al*., 2017) in embryos from the water. These results were not in agreement with the 100% mortality effect shown in Table 2, suggesting the BCF values are not necessarily presenting the aftermath of toxicants in embryos.

Differ from the combination of chemicals test, the combination of temperature + cadmium showed BCF values greater than 1 in both temperature treatments (Table 6). At a certain temperature level, the chorion structure gradually changes, thus making it more penetrable to metal (Hallare *et al*., 2005). This circumstance indicates that temperature elevates the uptake of cadmium in the embryos and magnifies the level higher than the values in water. The cadmium level in the combined test of 27 °C + 1.40 mg/L cadmium treatment was closed

**Table 6.** Bioconcentration factors (BCF) of zinc and cadmium in *A. testudineus* embryos

| Single exposure treatment | | | | |
|---|---|---|---|---|
| | | Cadmium (mg/L) | Embryos (mg/kg) | BCF[a] |
| | | Control | bdl (< 0.150) | na |
| | | 0.13 | bdl | na |
| | | 0.94 | bdl | na |
| | | 1.59 | bdl | na |
| | | 7.02 | 4.25 | 0.61 |
| | | 13.0 | 4.53 | 0.35 |
| | | Zinc (mg/L) | Embryos (mg/kg) | BCF[a] |
| | | Control | bdl (<2.20) | na |
| | | 2.63 | bdl | na |
| | | 4.20 | bdl | na |
| | | 13.00 | 12.12 | 0.93 |
| | | 49.6 | 36.33 | 0.77 |
| | | 146.00 | 124.00 | 0.84 |
| Combined exposure treatment | | | | |
| Temperature (°C) | | Cadmium (mg/L) | Embryos (mg/kg) | BCF[a] |
| 27 | + | 1.40 | 89.0 | 63.6 |
| 34 | + | 1.73 | 23.0 | 13.2 |
| Zinc (mg/L) | | Cadmium (mg/L) | Embryos (mg/kg) | BCF[a] |
| | Control | | bdl | na |
| 2.98 | + | 1.85 | bdl + bdl | na |
| 21.60 | + | 2.05 | 8.90 + bdl | 0.41 + na |
| 30.60 | + | 1.90 | 12.50 + bdl | 0.41 + na |
| 34.80 | + | 1.79 | 16.00 + bdl | 0.46 + na |

[a] BCF = embryos (mg/kg) / water (mg/L)
Room temperature (26.7 to 29.5 °C)
bdl – below detection limit
na – not available

to the single test of 1.59 mg/L cadmium (Table 3). The only difference was that the temperature in the combined test was kept constant in an incubator, while the temperature was fluctuated between 2 to 3 °C in a single test, in correspond to room temperature. Furthermore, BCF values can be calculated combined but not in single tests. This suggests that even at the optimum temperature for embryo growth, the constant temperature might accelerate the diffusion of cadmium into embryos. Hallare *et al*. (2005) discovered that cadmium uptake and bioconcentration could be measured in *Danio rerio* embryos regardless of constant low or high temperature. As such, the act of maintaining the temperature at a constant value potentially accelerates the bioconcentration level of cadmium in the *A. testudineus* embryos.

## CONCLUSION

Consequently, $LC_{50}$, LOEC, and NOEC values of zinc and cadmium showed that the embryos of *A. testudineus* were sensitive to metals. The trend of mortality is also consistent with the amount of zinc and cadmium concentration. The combination of constant temperature and cadmium could elevate cadmium uptake in embryos even in the best rearing temperature values (27 and 34 °C). For further verification, a combination of constant temperature and another type of can be tested in the future using embryos of this species. The experimental set-up for this study only thee of 50 mL beaker per treatment thus generating fewer wastes. Considering the limited space and resources to set up a new rearing facility, the embryos of *A*. *testudineus* could be easily established and used as an alternative test organism in the Southeast Asia region.

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## ETHICAL STATEMENT

The output of this research is mainly to conduct a preliminary trial in FET exposed to temperature, cadmium, and zinc using *A. testudineus* as an alternative embryotoxicity model in the Southeast Asia region. The research was conducted in Universiti Malaysia Terengganu (UMT) and approved by UMT Research Ethics Committees on November 26, 2020, with the number of animal ethics approval of UMT/JKEPHMK/2-2-/49.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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