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Embryonic toxicity of 3,4-dichloroaniline (3,4-DCA) on Javanese medaka (*Oryzias javanicus* Bleeker, 1854)

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ABSTRACT

Early-life exposure to toxic chemicals causes irreversible morphological and physiological abnormalities that may last for a lifetime. The present study aimed to determine the toxicity effect of 3,4-Dichloroaniline (3,4-DCA) on Javanese medaka (*Oryzias javanicus*) embryos. Healthy embryos were exposed to various 3,4-DCA concentrations for acute toxicity (5, 10, 25, 50, and 100 mg.L^{-1}) and sublethal toxicity (0.10, 0.50, 1.25, 2.50, and 5.00 mg.L^{-1}) for 96 h and 20 days respectively. Acute toxicity test revealed that the median lethal concentration (96h-LC$_{50}$) was 32.87 mg.L^{-1} (95 % CI = 27.90–38.74, $R^2 = 0.95$). Sublethal exposure revealed that 1.25 mg.L^{-1} at 3 days post-exposure (3 dpe) has a significant lower heartrate (120 ± 12.3 beats/min., $p < 0.01$), while at 7 dpe those exposed to 5 mg.L^{-1} (141.8 ± 8.3 beats/min) had significantly ($p < 0.01$) lower heart rate compared to other treatments. Likewise, at 13 dpe, 5.00 mg.L^{-1} (110.4 ± 17.3 beats/min) and 2.5 mg.L^{-1} (130.4 ± 8.3 beats/min) were significantly lower ($p < 0.001$) compared to control. None of the embryos in 5.00 mg.L^{-1} and 2.50 mg.L^{-1} treatment groups survived at the end of the experiment. The results indicated a concentration-dependent response. The lowest observed effect concentration (LOEC) that exerted developmental deformities was 0.5 mg.L^{-1}. Javanese medaka embryo have low sensitivity to acute toxicity of 3,4-DCA, but developmental abnormalities at sublethal concentrations were observed.

1. Introduction

Response to toxic substances at embryonic and larval stages varies with species [1]. Dichoroaniline (3,4-DCA) is categorized as a highly toxic and secondary poisonous chemical [2] and endocrine disruptor [3]. It is a highly persistent chemical as a result of its high stability and low volatility [4]. It is frequently used as phenylurea herbicides (diuron and linuron) and acylanilide herbicide (propanil) [4,5]. Chemical industries use 3,4-DCA as an intermediary in the production of azo dyes, paints, cosmetics [6–8]. Besides, this chemical has shown more toxic effects than these chemical products they are used for. It has an estimated half-life of 1000 days [6]. Concentrations of up to 567 μg.L^{-1} had been detected around the world in different environments [4,9]. 3, 4-DCA was three folds more toxic as compared to diuron in causing aneuploidy [10,11] and urothelial toxicity [11,12] in human cells. In rats, it caused kidney, liver and urinary bladder impairment [13]. 3, 4-DCA affects testicular enzymes of resulting in abnormal functions of the male reproductive system in rats [14]. 3,4-DCA affect morphological, biochemical, physiological and behavioural features of teleosts [11]. It disrupts endocrine activity in Nile tilapia (*Oreochromis niloticus*) [11,15]. The embryos and larvae are less sensitive compared to adult fish in response to endocrine disputing chemicals (EDCs) [16]. Previously, the fish embryotoxicity test indicated that the median effect concentration (EC$_{50}$) found were in the range of mg.L^{-1} [17,18]. Acute toxicity of adults may cause different outcomes in relation to size [19]. High toxic effect of 3,4-DCA to eleutheroembryo at the period of switching to an external food source is comparable in all egg-laying species [19]. It also suppressed the development of copepod larvae [20,21]. Therefore, there is a need to evaluate its potential toxicity to other life, especially aquatic animals [21].

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Javanese medaka (*Oryzias javanicus*) has been widely used in numerous researches as a model fish [22–24]. These include physical factor [25], heavy metals [23,26], genetics [27,27], fish pathology [25], organo-metals [28], and histological evidence of biocide exposure ([24], [29]). It is distributed around Peninsular Malaysia, Thailand, Singapore, and Indonesia. The species inhabit estuaries, freshwater, and marine, and are available throughout the year [22,23]. The advantages of wide salinity tolerance [30], zebrafish and medaka are observed to be excellent vertebrate-models for *in vivo* biological research [31], making it suitable for toxicity testing. Much research on toxic and genotoxic effects of 3,4-DCA on different organisms were conducted, the genotoxicity of this chemical that might arise as a result of degradation of pesticides was established [2]. To our knowledge, there are limited/no reports on the response of the embryo of this euryhaline fish, Javanese medaka to 3,4-DCA exposure. Therefore, this research aimed to evaluate the acute and sublethal effect of 3,4-DCA on embryonic development of Javanese medaka.

2. Materials and methods

2.1. Source of chemicals

3,4-Dichloroaniline (3,4-DCA); 98 % purity, CAS: 95−76-1 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulphoxide (DMSO); 99.9 %, CAS: 67−68-5 was obtained from Friedemann Schmidt Chemical, Malaysia.

2.2. Javanese medaka broodstocks culture and maintenance

Wild strain of Javanese medaka were collected from Sepang estuary (2°37′15.38″N, 101°42′38.33″E), Malaysia and maintained in recirculating system at suitable conditions (temperature; 26 ± 1℃, pH; 7.8–8.0, DO; 5.5–6.0 mg.L^{-1}, photoperiod; 14:10 h light/dark cycle) for more than one year in Javanese Medaka Mass Culture Laboratory, Department of Biology, Universiti Putra Malaysia. They were fed twice daily *ad libitum* with freshly hatched (24 h post-incubation) *Artemia salina* nauplii. Filters were washed, and water was changed every three weeks to minimize the removal of beneficial microbiota.

2.3. Preparation of concentrations and embryonic exposure

Stock concentration (100 mg.L^{-1}) of 3,4-DCA was prepared by dissolving it in 0.1 % (v/v) dimethylsulfoxide (DMSO) in dechlorinated tap water with some modification [32,33]. Newly spawned (< 5 hpf) stage 8 embryos were collected from F_2- parents of the wild stain [34]. The embryos were grouped into 7 treatments; 5 different test groups prepared from the stock and 2 control groups (solvent; 0.02 % (v/v) DMSO, and dilution water) in triplicates (n = 10) for all the experiments. For the acute toxicity test, 100, 50, 25, 10, and 5 mg.L^{-1} were used. While, 0.10, 0.50, 1.25, 2.50, and 5.00 mg.L^{-1} were used for subchronic toxicity test. The embryos were rinsed with dechlorinated tap water before transferred into 100 ml crystallization dishes containing various concentrations of the test chemical. Fertilized embryos (n = 210) were selected using a stereomicroscope (Olympus CX31 2D, Japan) and transferred into 24-well plates 2.0 mL of the test solutions. Thirty embryos replicated three times were individually placed into each well to avoid cross-contamination [35]. No change of exposure solutions throughout the acute toxicity test. But, 90 % of the solutions were changed with freshly prepared treatments after every 24 h [36] in subchronic exposure. The plate was incubated at 26 ± 1 °C, photoperiod; 14:10 light/dark cycle under 837-lux light intensity [32,33]. Preliminary range-finding tests were determined using various concentrations before the definitive tests.

2.4. Developmental changes

Heartbeat, hatching rate, survival rate and morphological anomalies were observed, been essential developmental endpoints [33,36]. Average heart rate (beats/min.) was counted at 3dpe (when heart started beating) (Ismail et al., 2014), 7 dpe (when structures were observed) 13 dpe (24 h for an average hatching) using stopwatch to evaluate the effect on the cardiac function of the developing embryo. Morphological abnormalities were observed and photographed using a Leica™ (Leica Microsystem, Germany). Hatching rate in each exposure group was observed daily from the first hatching (11 dpe) to 20 dpe. The proportion of surviving individual embryos during the period of exposure was determined from 1 dpe until the termination of the experiment.

2.5. Data analysis

Probit analysis [37] using log concentration-response using variable slope, and minimum and maximum effects were 0% and 100 %. Shapiro-Wilk normality test and one-way ANOVA were determined (for parametric) using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, USA) at 95 % confidence interval and $p < 0.05$. Values = mean ± SEM (error bars).

3. Results

Acute toxicity indicates that the median lethal concentration at 96hpe (96h-LC_{50}) of 3,4-DCA on Javanese medaka embryos was 32.87 mg.L^{-1} (95 % CI, 27.90–38.74, $R^2 = 0.95$) while 10 % mortality was 13.91 mg.L^{-1} (96h-LC_{10}) indicating low acute embryonic lethality of the chemical to the test organism (Fig. 1).

The lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) for acute were 2.5 mg.L^{-1} and 1.25 mg.L^{-1}, respectively. Javanese medaka embryos showed low sensitivity to acute exposure (96h-LC_{50}) of 3,4-DCA in comparison with some adult fish and other species (Table 1).

The average heartbeats/minute (HBpM) of 3,4-DCA-exposed Javanese medaka embryos compared with control group showed that at 3 days post-exposure (3 dpe) it was significantly lower in 2.50 mg.L^{-1} (120 ± 12.3 HBpM, $p < 0.01$), individual exposed to the highest concentration (5.00 mg.L^{-1}) observed on 7 dpe showed a significantly lower number of beats 141.8 ± 8.3 HBpM, $p < 0.01$). The HBpM at 13 dpe indicated that 2.50 mg.L^{-1} and 5.00 mg.L^{-1} exposed-embryos were significantly lower, (130.4 ± 8.3 HBpM, $p < 0.001$) and (110.4 ± 17.3 HBpM, $p < 0.001$) respectively. The LOEC and NOEC for the heartbeat at 13dpe were 1.25 and 2.50 mg.L^{-1} respectively (Fig. 2).

First hatching rate (%) was recorded in control and solvent groups at

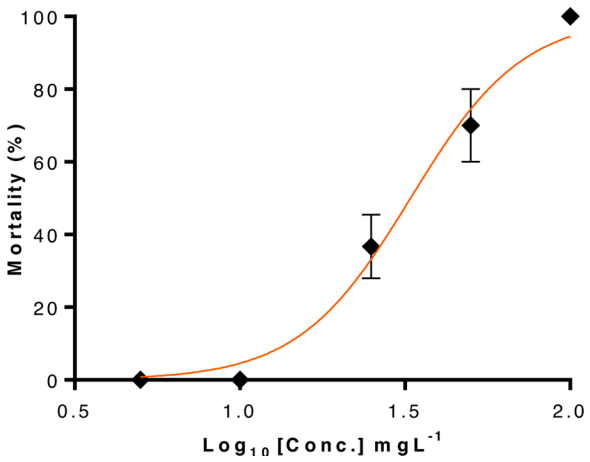

Fig. 1. Concentration-response curve of Javanese medaka embryos exposed to 3,4-DCA.

Table 1
Acute toxicity of 3,4-DCA on some aquatic species.

Species	Organism (life stage)	96h-LC_{50} (mgL^{-1})	Reference
Zebrafish (*Danio rerio*)	Fish (adult)	7.17 – 12.9	EMJRC (2017) [59]
Fathead minnow (*Pimephales promelas*)	Fish (adult)	6.99	Call et al. (1987) [57]
Rainbow trout (*Oncorhynchus mykiss*)	Fish (adult)	1.94	Hodson [38]
Guppy (*Poecilia reticulate*)	Fish (adult)	8.90	Adema and Vink [39]
Perch (*Perca fluviatilis*)	Fish (adult)	3.10	Schäfers and Nagel [19]
Brine shrimp (*Artemia salina*)	Crustacean (adult)	14.00	Adema and Vink [39]
Japanese medaka (*Oryzias latipes*)	Fish (adult)	11.00	EMJRC, 2017 [59]
Javanese medaka (*Oryzias javanicus*)	Fish (embryo)	32.87	This study

11 dpe with 10 % and 3.3 % correspondingly. None of the individual embryos (0%) exposed to 2.50 and 5.00 $mg.L^{-1}$ hatched throughout exposure (20 days) to sublethal concentrations of the test substance (Fig. 3).

Embryos exposed to highest concentration (5.00 $mg.L^{-1}$) had a maximum average survival rate of 93.3 % for 11 dpe, subsequently, a decrease in survival rate was observed in all other groups except control and solvent groups. At 18 dpe, only 3.3 % of 2.50 $mg.L^{-1}$ exposed-group survived, while 0% survival rate in 5 $mg.L^{-1}$ exposed-embryos (Fig. 4).

Coagulated embryos were observed in 5.00 $mg.L^{-1}$ at 7 dpe. However, on 12 dpe 2.50 $mg.L^{-1}$ exposed-group showed some morphological abnormalities such as pericardiac oedema, yolk-sac oedema, tail curvature, scoliosis and abnormal head development (Fig. 5).

At one day post hatched (1 dph), the frysac-larvae exposed to 0.50 $mg.L^{-1}$ (the LOEC for anomalies) showed a sign of tail curvature, yolk-sac and pericardial oedema, tissues disintegration and scoliosis at 13 dpe to 1.25 $mg.L^{-1}$. Furthermore, slight yolk-sac oedema was observed at 15 dpe in 0.50 $mg.L^{-1}$ exposed frysac-larvae (Fig. 6).

4. Discussion

Embryo and larvae of fish are very sensitive to exogenous toxicants, which affect their morphological features [1]. Chemical industries ranging from pesticides, pharmaceutics, cosmetics, antifouling paints, clothing and others that use 3,4-DCA increase in proportionate to human

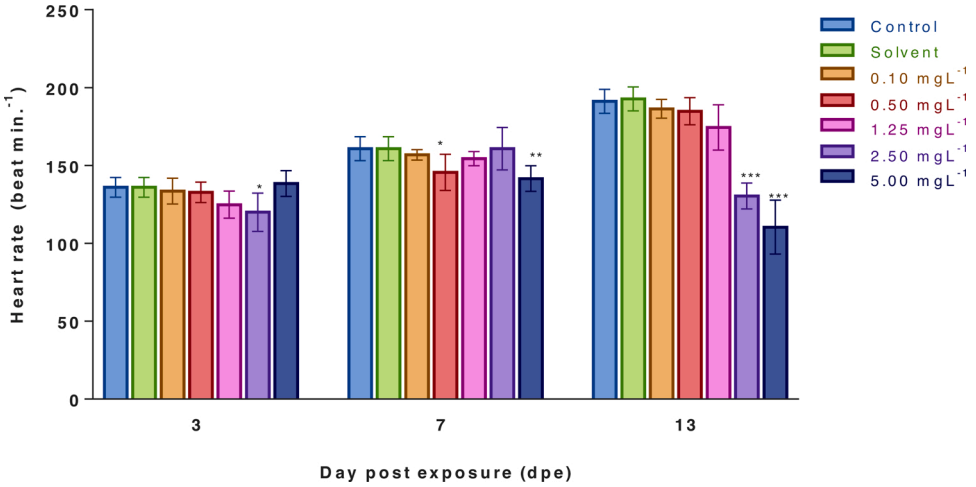

Fig. 2. Heart rate (n = 3) of Javanese medaka embryo at 3, 7 and 13 dpe to 3, 4-DCA. *, **, *** indicates a significant difference with control within each group at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

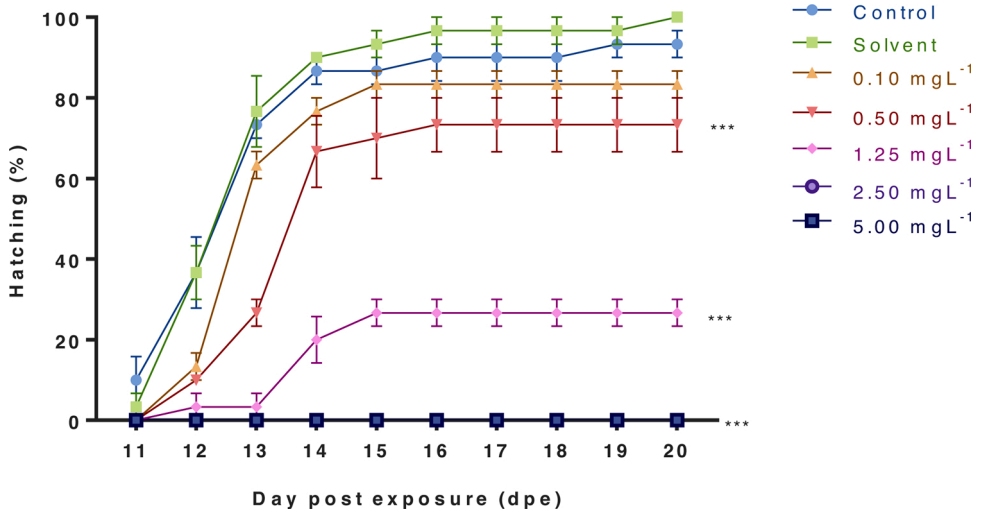

Fig. 3. Hatching rate (n = 3) of Javanese medaka embryos at different days after exposure to sublethal concentrations of 3,4-DCA. *** indicates a significant difference (20 dpe) with control within each group at $p < 0.001$.

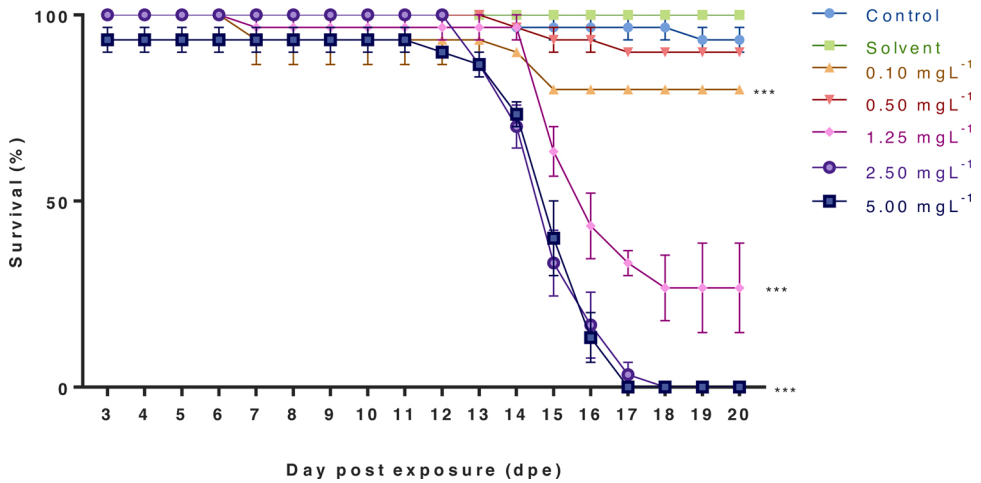

Fig. 4. Survival rate (n = 3) of Javanese medaka embryos at different days after exposure to 3,4-DCA. *** indicates a significant difference (20 dpe) with control within each group at $p < 0.001$.

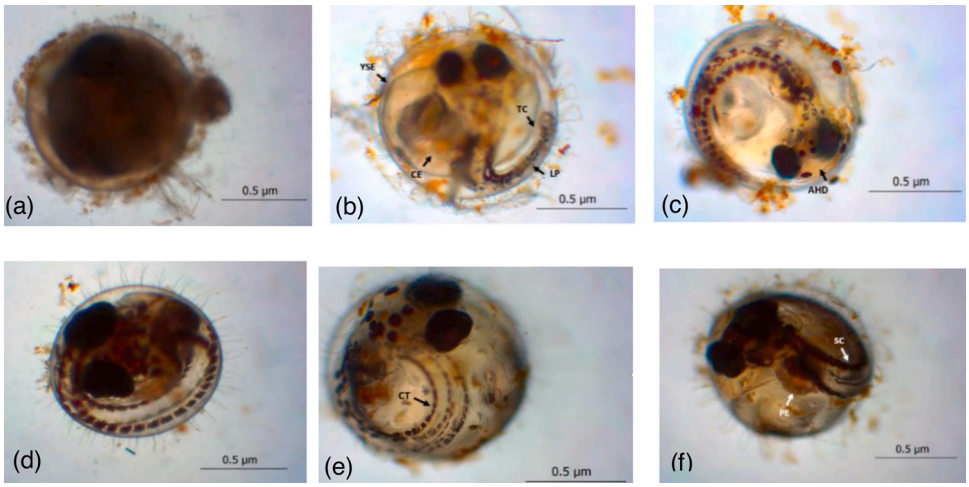

Fig. 5. Malformations in Javanese medaka embryos exposed to 3,4-DCA (a) coagulated embryo in 5.00 mg.L^{-1} (7 dpe) (b) and (c) 2.50 mg.L^{-1} (12 dpe) (d) normal embryo (12 dpe) (e) and (f) 2.50 mg.L^{-1} and 5.00 mg.L^{-1} respectively (13 dpe). YSE = yolksac oedema, LP = low pigmentation TC = tail curvature, AHD = abnormal head development PE = pericardial oedema, SC = scoliosis, CT = coiled tail.

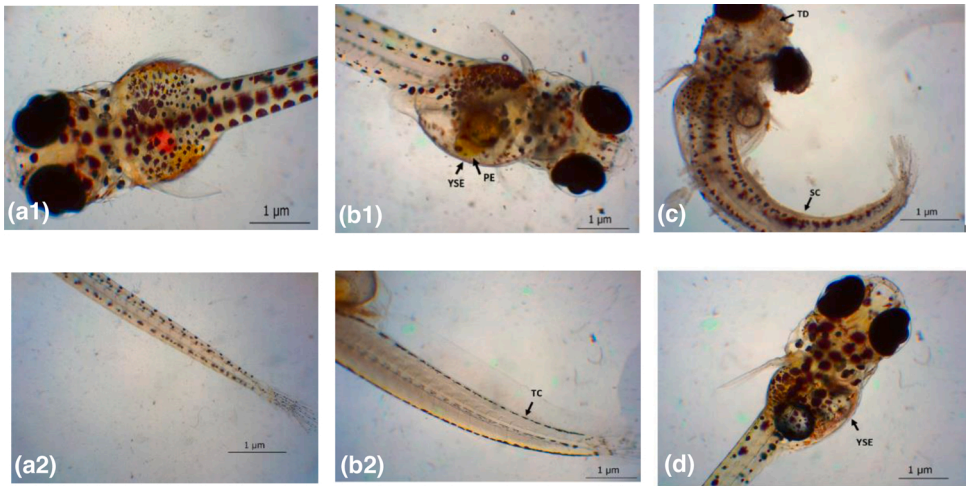

Fig. 6. Deformities in Javanese medaka larvae after exposure to 3,4-DCA (a) Normal (control) at 1dph (b) 0.50 mg.L^{-1} (1dph) (c) 1.25 mg.L^{-1} (13 dpe) (d) 0.50 mg.L^{-1} exposed larvae 15dpe. YSE = yolksac oedema, CE = cardiac oedema, TC = tail curvature, TC = tail curvature, TD = tissue disintegration, PE = pericardial oedema, SC = scoliosis.

need which endangered human and wildlife health. Dichloroaniline is known to be toxic to fish at 96 h LC_{50} [38,40]. 3,4-DCA is a precursor for the synthesis and a degradation product of some herbicides and is commonly present in the environment [14]. In the principles of animal research, fish embryo toxicity test (FET) represents the refinement of the 3Rs (replacement, refinement, and reduction) of animals in research. Complaints to the application of the FET have mainly been based on the hypothetical lack of biotransformation ability and the assumption that highly lipophilic and/or high molecular weight substances might not able to penetrate the embryo due to shielding effect of the chorion [41]. The chorion of the medaka egg becomes so rigid after fertilization that it cannot be infiltrated with a fine glass needle or micropipette [42]. This finding revealed the low sensitivity of embryonic stages of Javanese medaka to acute toxicity (96 h) of 3,4-DCA. However, More tolerance to 3,4-DCA was observed in molluscs; pond snail (*Lymnaea stagnalis*) and zebra mussel (*Dreissena polymorpha*) after exposure at stages of first cleavage egg (48 h $LC_{50} > 32$ mg.L^{-1}) or adult ($LC_{50} = 22$ mg.L^{-1}) [39].

Subsequently, the 96 h LC_{50} is age/life stage, species, time and the chemistry of the substances the organism was exposed to. The LC_{50} of Javanese medaka embryos is almost 15 folds as compared to zebrafish (*Danio rerio*) embryo and other adults of various species which might be as a result of low chemical penetration through the chorion that varies with physicochemical properties of the compound [43] and electrostatic attraction between chemicals and the chorion [44]. These factors show that the chorion is selectively permeable to chemicals under some conditions [45]. The higher mortality of embryos in 0.1 mg.L^{-1} compared to 0.5 mg.L^{-1} in (Fig. 4) might be attributed to non-monotonic dose-response (NMDR), which is characterized by low dose initiation and high dose inhibition associated by endocrine disrupting chemicals (EDCs) as observed by [46,47]. Renieri et al. [48] also reported a non-monotonic mortality response in zebrafish exposed to low and high concentrations of cadmium. In a combined-exposure of rainbow trout cell lines to two pharmaceuticals, Bain and Kumar [49] observed a non-monotonic dose-response (NMDR). Likewise, Zheng et al. [50] reported a NMDR development of ovary in rare minnow (Gobiocypris rarus) to bisphenol A. So far, reported LOECs for survival and sublethal effects in 48 h old embryos are 2.01 mg.L^{-1} [35]. Scheil et al. [51] observed that LOEC for deformations was 1.0 mg.L^{-1} in the 96 h embryo test and 0.25 mg.L^{-1} in the subchronic test; LOEC for mortality. In a similar study, Yuen et al. [16] reported that di-(2-ethylhexyl)-phthalate (DEHP), an EDC might have exerted a molecular effect on Javanese medaka embryos, which resulted in mortality during embryogenesis.

Using the immobilization test with 3,4-DCA, water flea (*Daphnia magna*) showed a highly effective response (EC_{50}) value of 226 mg.L^{-1} [52,53].

The major developmental abnormalities imposed by 3,4-DCA in this result were age and concentration-dependent, as the highest concentration and second-highest concentration showed similar abnormalities five days later. This is in line with the findings of Saeed et al. [18] who observed that 3,4-DCA can cause heart and yolk-sac oedema in embryo in a similar trend. A developing embryo that exceeded 15 days post-spawned unhatched, was considered as a slow-developing embryo [58, 22]. In normal embryonic development of medaka, the body is ventrally curved, as the tail elongates toward the head [22]. However, the normal heart rate for Japanese medaka (*Oryzias latipes*) range between 106–113 HBpM for 3 days [54]. In this study, the normal heart rate of Javanese medaka ranged was 120–160 HBpM which is slightly higher for this species. The decrease in heart rate of embryos exposed to 1.25 and 2.5 mg.L^{-1} 3,4-DCA at 3 dpe compared other groups, also implies NMDR. The NMDR, which is one of the major challenges of environmental, and health risk assessment of chemicals, was observed in radiation and EDCs induced toxicity [16]. Non-monotonicity is becoming clearer in toxicological research with low and high doses showing similar response pattern [48]. In a chronic fish embryonic exposure (96 hpf) to 3,4-DCA, a significant increase of cardiac and yolk-sac oedema at $\geq$ 1 mg.L^{-1} compared to control. At 14 dpe, pigmentation and abnormalities were the most sensitive sublethal endpoints in zebrafish (*Danio rerio*) embryo [35]. Common goby (*Pomatoschistus microps*) exposed to 0.5–1.49 mg.L^{-1} 3,4-DCA exerted sublethal effects after only 96 hpe. 3,4-DCA sensitivity dependents highly on species. But, oyster (*Crassostrea gigas*) exposed to 3,4-DCA show no embryotoxicity at concentrations up to 5 mg. L^{-1} [53]. In Crucian carp (*Carassius auratus*), 3,4- DCA caused necrosis, liver lesions, and degeneration that could result in oxidative stress and lipid peroxidation from the concentration of 0.2 mg.L^{-1} [21].

The decrease in some of the developmental features observed in this study is also similar to the finding of Zhu et al. [17] who reported embryo-larval developmental toxicity in rare minnow (*Gobiocypris rarus*) exposed to 3,4-DCA by reducing the rate of survival, increasing malformation and alterations in heartbeat. Schäfers & Nagel found that hatching rates of 85 % indicate good experimental conditions and hatched larvae showed oedema and malformations at 20 and 100 μg.L^{-1} [19]. In similar embryo-larval exposure, Sumithion, an organophosphate pesticide affected development of zebrafish [1]. Alteration in morphological features implies a delay in development, but a change of the body axis was observed in some embryos [55]. The concentration that exerted effect in this research is a bit above the ones used by Sardo et al. [56] with a sublethal effect of 3,4-DCA which ranged between 0.30–1.40 mg.L^{-1}. But, oyster (*Crassostrea gigas*) exposed to 3,4-DCA showed no embryotoxicity at concentrations up to 5 mg.L^{-1} [53].

5. Conclusion

In this study, the toxicity of 3,4-DCA on developing embryo of Javanese medaka was revealed. The finding showed low sensitivity of the test embryo to acute toxicity of 3,4-DCA. However, prolonged exposure to sublethal concentration has developmental anomalies, reduced hatchability and survival rates that might be liked to endocrine disruption by the test chemical. This species is new model fish and euryhaline, a comparative toxicity test of 3,4-DCA and other toxic substances at different salinity levels would give more insight on test results. Dechorionation could also provide the clearest approach to examine the role of the chorion for chemical toxicity, thereby exposing the unprotected embryo. Further studies to evaluate biochemical and oxidative stress markers in developing an embryo would give more details to back this finding.

CRediT authorship contribution statement

Musa Adamu Ibrahim: Conceptualization, Methodology, Resources, Validation, Writing - original draft. **Syaizwan Zahmir Zulkifli:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Project administration. **Mohammad Noor Amal Azmai:** Validation, Supervision. **Ferdaus Mohamat-Yusuff:** Writing - review & editing. **Ahmad Ismail:** Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.08.011.

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