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# Effects of Reactive Red 239 on Developing Zebrafish (*Danio rerio*) Embryos

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## ABSTRACT

The acute effects of the well-known and widely used mono azo-dye, Reactive Red 239 (RR 239) were investigated using the sixth cleavage stage of zebrafish embryos as a biological experimental model. Toxicity was evaluated through three endpoints—survival rate, hatching rate and normality of embryo development. In order to find the key parameter relating to survival of RR 239-exposed embryos, the relationships between survival and concentration or malformations were studied using a cuboidal equation and multiple linear regression. The results revealed that exposure to RR 239 during 96 hr gave a median lethal concentration value of 1,579 ± 25 $mg.L^{-1}$ which could lead to the classification of RR 239 as a nontoxic textile dye for aquatic organisms and was expressed as an effective concentration value of 1,536 ± 36 $mg.L^{-1}$ for the assay. However, exposure to RR 239 with a higher concentration than 500 $mg.L^{-1}$ reduced the embryonic survival rate as well as the hatching rate on the one hand and stimulated various morphological malformations on the other hand. Cuboidal equations showed that the concentration, yolk sac edema and hatching play important roles in survival, with hatching having the greatest effect of these factors. The results indicated that the probable effects of released effluent containing RR 239 on natural aquatic organisms should be further explored.


**Keywords:** Azo dye, toxicity, zebrafish embryo, morphological malformation, survival

## INTRODUCTION

Azo dyes, especially reactive dyes showing favorable characteristics for textile dyeing, have been widely used in various industries (Puvaneswari *et al.*, 2006; Karadag *et al*., 2007; Cirstovao *et al.*, 2009; Tavares *et al*., 2009). Some of these dyes is lost during the dyeing process and ultimately about 2−50% is released into the environment (Chen, 2002; Ozdemir *et al*., 2004). Due to their high solubility and removal difficulty, these dyes cause serious environmental problems with both aesthetic and toxic effects (Armagan *et al*., 2003). Many azo dyes and their metabolites of aromatic amines cause blocking of oxygen binding by hemoglobin, are toxic to aquatic life and mutagenic to humans (Gupta, 2005; Meric *et al*., 2005; Zee and Villaverde, 2005; Tavares


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*et al*., 2009). Aquatic organisms are probably the most appropriate biological indicators to assess the quality of water discharged by the textile industry (Kanu and Achi, 2011; Karci, 2014).

Zebrafish (*Danio rerio*) embryos have been widely utilized for toxicity testing due to various advantages including both rapid and synchronous development and optical transparency resulting in easy observation of any malformation in the internal organs (Barros *et al*., 2008; Bai *et al*, 2010). Zebrafish embryos ranging from 5 to 7 d after fertilization are also an ethically acceptable vertebrate model based on European Union (EU) Directives (Belanger *et al*., 2010; Ali *et al*., 2011; Strahle *et al*., 2011). Moreover, zebrafish genes are approximately 75% homologous to human genes leading to their use as a surrogate for humans in various toxicity types (Barbazuk *et al*., 2000; Hill *et al*., 2005).

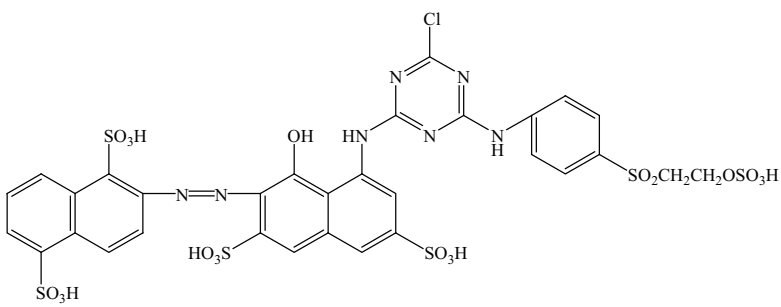
Reactive Red 239 (RR 239) is a well-known mono-azo dye that has been intensively used to dye fabric in various textile industries (Liu and Chiou, 2005). However, toxic information on RR 239 has not been widely reported, especially its toxicity on aquatic organisms such as fish embryos. In order to ensure the safe use of this dye according to the requirements of EU legislation known as REACH (European Union Commission, 2006), the toxic effects of the well-known and widely used mono-azo dye, RR 239, were therefore investigated in this study through three endpoints consisting of the survival rate, hatching rate and normality of embryo development. Cuboidal equations and multiple linear regression to reveal the relationships between survival and concentration or malformations were studied in this work in order to investigate the key parameter relating to the survival of embryos exposed to RR 239.

## MATERIALS AND METHODS

### Chemicals and test solution

Reactive Red 239 (RR 239) was kindly provided by DyStar (Thai Co., Ltd). The chemical structure and related information are shown in Table 1 (Tavares *et al*., 2008). Test solutions of RR 239 concentrations (0−4,000 mg.$L^{-1}$) were freshly prepared using 50% distilled water and made up by diluting rearing water.

**Table 1** Chemical structure and related information of mono azo-dye, Reactive Red 239 (Tavares *et al*., 2008).

| Topic | Information |
|---|---|
| Color index | Reactive Red 239 |
| Abbreviation | RR 239 |
| Trade name | Remazol Brilliant Red 3BS |
| Chemical structure |  |
| $\lambda_{max}$ (nm) | 542 |

$\lambda_{max}$ = Wavelength of maximum absorbance.

**Zebrafish husbundary and egg collection**

Zebrafish embryos aged 5−7 d after fertilization, provided an ethically acceptable vertebrate model under EU directives (Belanger *et al*., 2010; Ali *et al*., 2011; Strahle *et al*., 2011) and were used as the biological model throughout the study. Healthy male and female zebrafish aged 3−6 mth were cultured separately in glass aquaria under a natural dark/light cycle. The fish were fed daily with ultrafine powdered food containing 35% protein and *Artemia* nauplii. Physical and chemical water quality parameters for housing and breeding were controlled (temperature = 28.63 ± 1.70 °C; pH = 8.18 ± 0.08; conductivity = 306.50 ± 3.0 $\mu S.cm^{-1}$; ammonia-nitrogen = 0.31 ± 0.07 $mg.L^{-1}$ $NH_3$-N; dissolved oxygen = 6.86 ± 0.56 $mg.L^{-1}$ $O_2$; alkalinity = 78.65 ± 1.83 $mg.L^{-1}$ $CaCO_3$, and hardness = 121.40 ± 4.00 $mg.L^{-1}$ $CaCO_3$).

In order to produce fertilized eggs, two males were mated with one female under the previously described culture conditions and fertilized eggs were collected within 1 hr after spawning and rinsed several times with rearing water. At least 80% of fertilization of eggs from spawn indicated the validity of egg production and the collected eggs were subsequently used in the designed experiment.

**Acute toxicity test of RR 239**

Fish embryos used in a toxicity test are limited to the seventh cleavage stage or 128-cell stage of cell division (Lammer *et al*., 2009). In the current study, embryos at the sixth cleavage stage (64-cell stage) were used. Each of 20 embryos selected by random sampling was cultured separately in the presence of 2 mL of the designated concentration of RR 239 (0−4,000 $mg.L^{-1}$). Embryos were maintained at 26 ± 1 °C with a lighting schedule of 12 hr light and 12 hr dark. All experiments were conducted in quadruplicate. Embryo mortality was observed and recorded during 96 hr and determined using each of four different criteria (coagulation, failure of tail detachment, somite malformation and lack of heartbeat). Hatched larvae were checked and recorded.

**Morphological analysis**

The 64-cell stage of fertilized eggs was used in the embryonic malformation investigation. Each of 10 embryos was exposed to 20 mL of designed concentration of RR 239. All experiments were performed in quadruplicate. Morphological malformation based on a comparison with normal development (Kimmel *et al*., 1995) was observed, counted and photographed every 12 hr using a light microscope equipped with a digital camera.

**Statistical analysis**

The median lethal concentration ($LC_{50}$) and the effective concentration ($EC_{50}$) values defined as the concentration of RR 239 of zebrafish embryo to 50% of the control were estimated from a dose-response curve. Mean differences in the hatching rate and malformation after exposure to RR 239 were determined using a one-way analysis of variance followed by Duncan's new multiple range test (DMRT) with significance tested at the $P < 0.05$ level. The relationships between the survival and concentration or malformations were constructed and analyzed using cuboidal equations; then, multiple linear regressions were developed to find the key parameter influencing survival. All data were presented as mean ± SE.

## RESULTS

**Acute toxicity of RR 239**

The effect of RR 239 on the survival rate of embryos after exposure for 96 hr is shown in Figure 1. Variability in the dose-response manner of fish embryo exposed to RR 239 was observed with an initial phase of nontoxic effect up to 500 $mg.L^{-1}$ and thereafter, higher concentrations of this compound exhibited rapidly decreasing embryonic survival. The calculated $LC_{50}$ of RR 239 on fish embryo (1,579 ± 25 $mg.L^{-1}$) was obtained from curve-fit analysis.

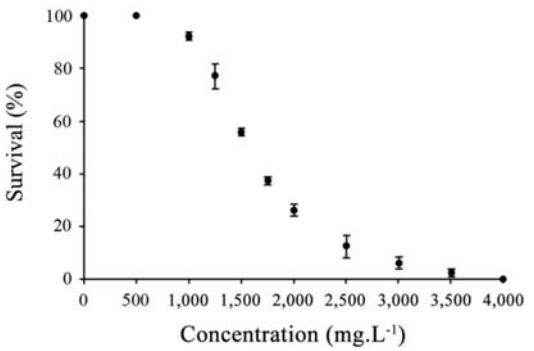



**Figure 1** Survival rate of zebrafish embryos after 96 hr exposure to mono azo-dye, Reactive Red 239. Error bars indicate mean ± SE.

**Hatching rate**

Similar to the survival rate of zebrafish embryos, RR 239 reduced the hatching rate of embryos in a concentration-dependent manner (Figure 2). The calculated $EC_{50}$ value of RR 239 obtained from curve-fit analysis was 1,536 ± 36 mg.L$^{-1}$. Exposure to 500 mg.L$^{-1}$ of RR 239 exhibited a nonsignificant effect on the hatching rate of fish embryos. However, a reduction in the hatching rate as a function of the RR 239 concentration was observed after the zebrafish embryos were exposed to higher concentrations of RR 239.

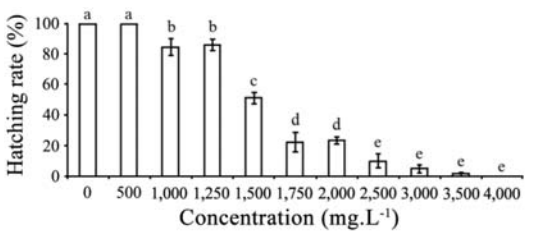



**Figure 2** Hatching rate of zebrafish embryos after 96 hr exposure to mono azo-dye, Reactive Red 239. Different lowercase letters above a bar indicate a significant difference ($P < 0.05$). Error bars indicate mean ± SE.

**Morphology of RR239-exposed zebrafish embryos**

Compared with the control, exposure to 500 mg.L$^{-1}$ of RR239 revealed no toxic effects as determined by morphological malformation, with no deformities observed and a well developed tail, head and a normal body structure were observed (Figures 3−5). Morphological malformations were found after exposure to higher concentrations of RR239 (Figures 3−5). Head and eye hypoplasia, tail malformation and cardiac edema (Figure 3) were observed after 24 and 48 hr of exposure to 2,500 and 3,000 mg.L$^{-1}$ of RR 239. In addition, deformities, yolk sac edema, spine bending and curved tail were found in embryos exposed to 1,250−2,500 mg.L$^{-1}$ for 72 and 96 hr (Figures 4b−f and Figures 5c−m).

Compared to normal development in the control, exposure to RR 239 caused several morphological malformations in zebrafish embryos. As shown in Figures 6a−d, RR 239 with a concentration range of 1,500−2,500 mg.L$^{-1}$ caused significantly increased yolk sac edema while other deformities were found to be not significant. Moreover, exposure to RR239 during the post-hatched period caused greater embryonic abnormalities than during the pre-hatched period. Yolk sac edema was the highest morphological malformation (26.88 ± 5.65%) followed by pericardial edema (12.13 ± 2.56%), bent spine (6.38 ± 1.40%) and tail malformation (0.75 ± 0.42%), respectively. It was also found that some morphological embryo deformation could lead to embryo mortality; in particular, the death of embryos with yolk sac edema was observed within 14 d.

**Survival**

In order to find the key parameter affecting the survival of zebrafish embryos exposed to RR 239, the relationships between survival and the concentration or malformations was studied using cuboidal equations. Survival

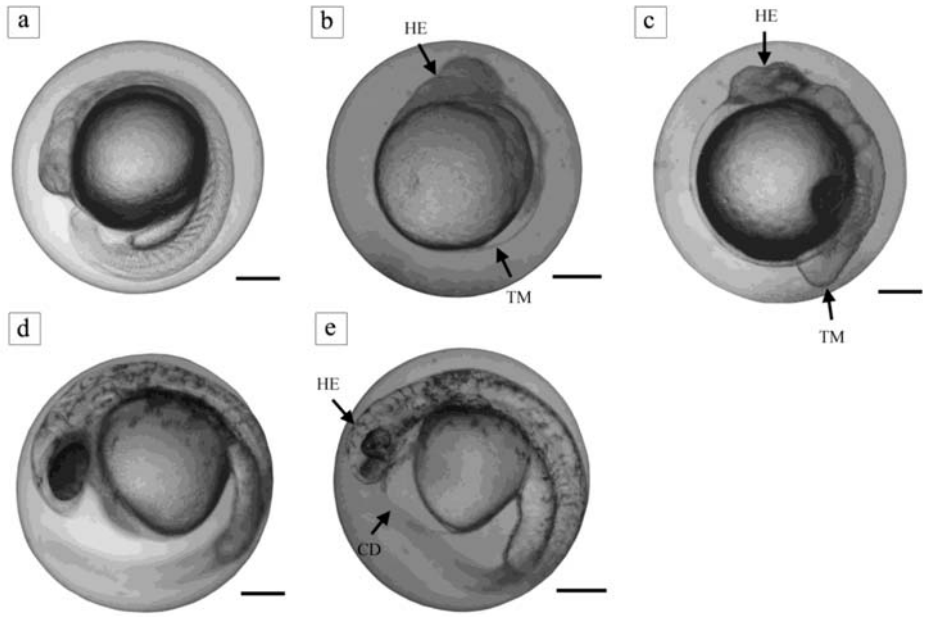



**Figure 3** Morphology of embryos exposed to mono azo-dye, Reactive Red 239 at: (a-c) 24 hr and (d-e) 48 hr; (a and d) control, (b and e) 2,500 mg.L$^{-1}$ and (c) 3,000 mg.L$^{-1}$. HE = Head and eye hypoplasia; TM = Tail malformation; CD = Cardiac edema. Scale bar = 200 µm.

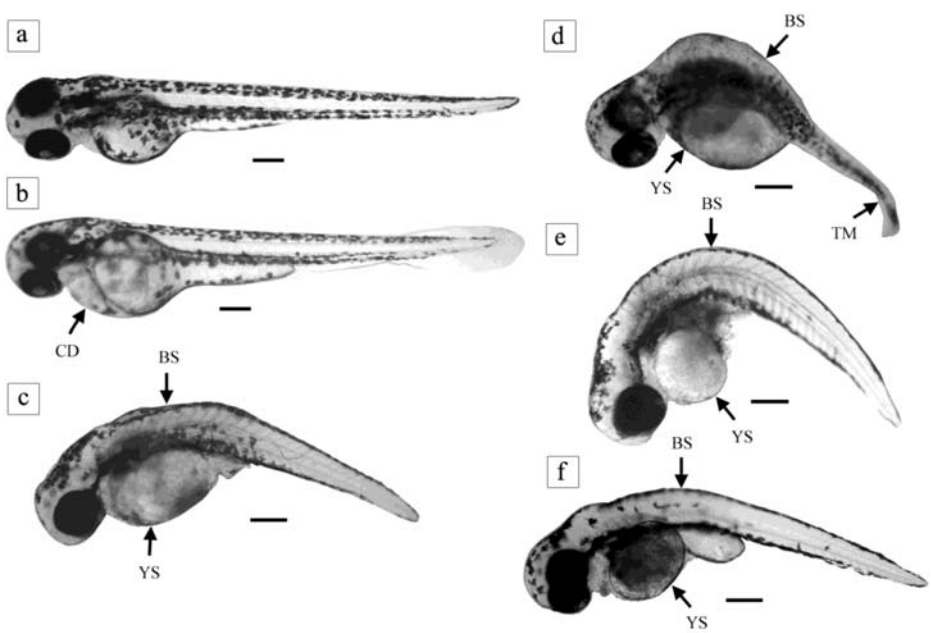



**Figure 4** Control embryo (a) and embryos exposed to mono azo-dye, Reactive Red 239 (RR 239) for 72 hr causing: (b) cardiac edema; (c-f) yolk sac edema and bent spine malformations such as (c-f) kyphosis; with RR 239 concentration at (a) 0 mg.L$^{-1}$ (control); (b) 1,000 mg.L$^{-1}$, (c) 1,250 mg.L$^{-1}$; (d) 1,500 mg.L$^{-1}$; (e) 1,750 mg.L$^{-1}$; and (f) 2,000 mg.L$^{-1}$. CD = Cardiac edema; BS = Bent spine; YS = Yolk sac edema; TM = Tail malformation. Scale bar = 200 µm.

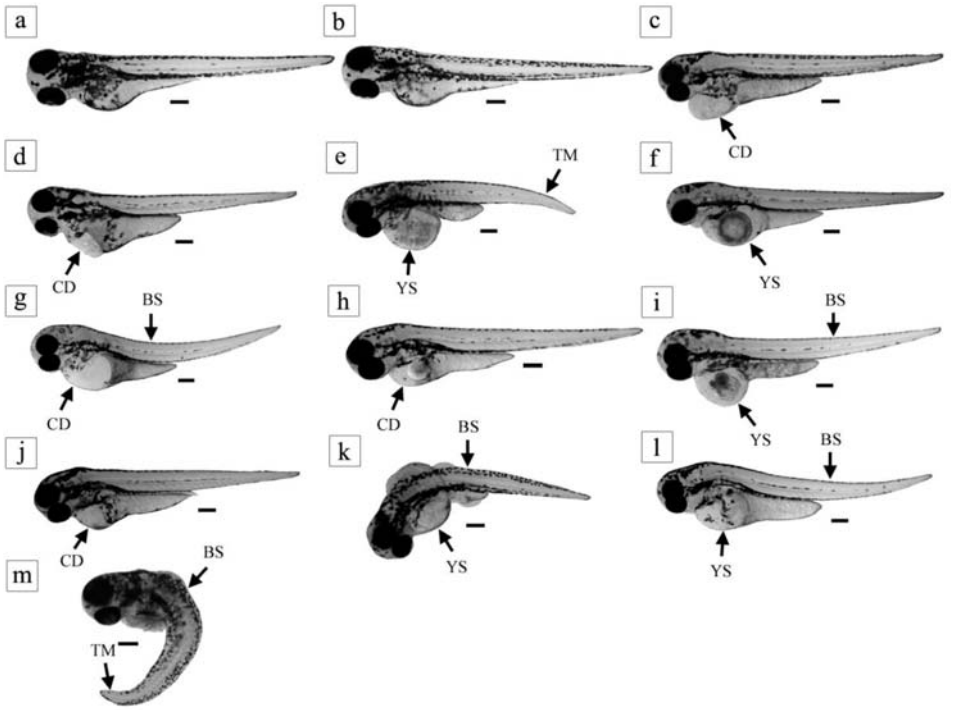



**Figure 5** Zebrafish embryos after exposure to (a) control and mono azo-dye, Reactive Red 239 (RR 239) at (b) 500 mg.L$^{-1}$; (c) 1,000 mg.L$^{-1}$; (d and e) 1,250 mg.L$^{-1}$; (f and g) 1,500 mg.L$^{-1}$; (h and i) 1,750 mg.L$^{-1}$; (j and k) 2,000 mg.L$^{-1}$; and (l and m) 2,500 mg.L$^{-1}$ for 96 hr. RR239-exposed embryos caused: (c, d, g, h and j) cardiac edema, (e, f, i, k and l) yolk sac edema, bent spine and (e and m) tail malformation. Observed bent spine in this study included (k) kyphosis, (g, i and l) lordosis and (m) scoliosis. CD = Cardiac edema; BS = Bent spine; YS = Yolk sac edema; TM = Tail malformation. Scale bar = 200 μm.

exhibited a significant correlation ($P < 0.01$) to the concentration, yolk sac edema and hatching while no relationship was found with bent spine, tail malformation and cardiac edema (Table 2).

Additionally, multiple linear regression of survival with the concentration, yolk sac edema and hatching, produced Equation 1:

$$\text{Survival} = 31.077 - 0.008\ \text{concentration} - 0.046\ \text{yolk sac edema} + 0.730\ \text{hatching} \quad (1)$$

Which was significant ($P < 0.01$) with a correlation coefficient ($R^2$) of 0.988. The obtained equation revealed that hatching had the greatest impact on survival compared to the test concentration and yolk sac edema.

## DISCUSSION

The acute effect of RR 239, a widely used reactive mono-azo dye, was investigated using zebrafish embryos to evaluate toxicity and to ensure the safe use of this dye according to the objectives of REACH. Compared with the USEPA (United States Environmental Protection Agency, 2012), the observed $LC_{50}$ of RR 239 with a value higher than 100 mg.L$^{-1}$ indicated that RR 239 can be classified as a nontoxic compound to aquatic organisms. The results observed in this study also revealed that exposure to 500 mg.L$^{-1}$ of RR 239 produced no significant effects on both the

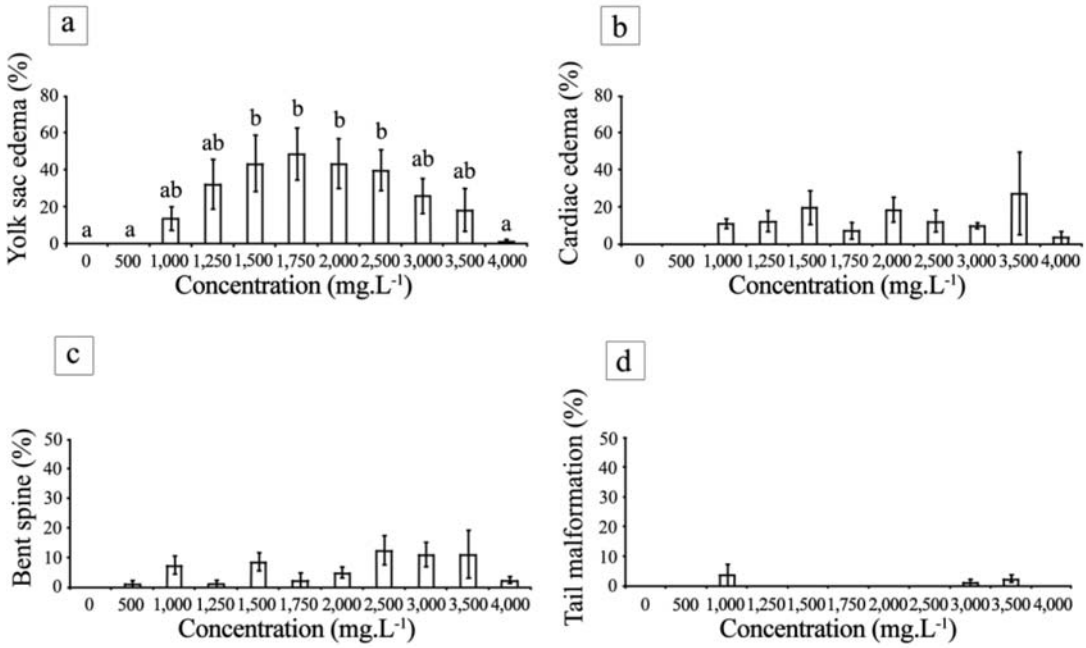



**Figure 6** Percentage of each morphological malformation after 96 hr of mono azo-dye, Reactive Red 239 exposure. Different lowercase letters above a bar are significantly different ($P < 0.05$). Error bars indicate mean ± SE.

**Table 2** Relationship between survival and concentration or malformations.

| Regression (X) | $R^2$ | Sig. | $b_0$ | $b_1$ | $b_2$ | $b_3$ |
|---|---|---|---|---|---|---|
| Concentration | 0.982 | ** | 3826.26 | -121.85 | 2.20 | -0.01 |
| Yolk sac edema | 0.942 | ** | 10.08 | 2.22 | -0.03 | 0.0001 |
| Hatching | 0.986 | ** | 1.69 | 0.25 | 0.02 | -0.0001 |
| Bent spine | 0.335 | ns | 8.70 | -0.05 | -0.00004 | -0.000001 |
| Tail malformation | 0.160 | ns | 1.45 | -0.12 | 0.003 | -0.00002 |
| Cardiac edema | 0.413 | ns | 14.10 | -0.20 | 0.01 | -0.00009 |

Regression equation: Survival = $b_0 + b_1X + b_2X^2 + b_3X^3$.

$R^2$ = Coefficient of determination; Sig = Significance level: ** = $P < 0.01$; ns = Not significant ($P > 0.05$).

survival and hatching rates of zebrafish embryos. However, reactive azo dye in textile dyehouse wastewater streams with concentrations between 5 and 1,500 mg.L$^{-1}$ have been reported (Gottlieb *et al*., 2003). Therefore, the loading in waste water containing RR 239 into aquatic systems should take note of the findings in this study that RR 239 at concentrations of higher than 500 mg.L$^{-1}$ could affect the survival, hatching and morphology of embryonic aquatic life. The failure of embryos to hatch might have been due to the reduction of embryonic movements caused by RR 239 resulting in the unusual distribution of hatching enzymes and subsequently the embryo could not break the non-digestible outer part of the egg envelope (Hallare *et al.*, 2005; McCormick *et al.*, 2010). Lefebvre *et al.* (2004) reported that a severe reduction in the heart rate, lack of movement and imbalance in the osmotic pressure were causes of edema and body curvature. However, the mechanism by which RR239 influences morphological malformations is not presently known.

## CONCLUSION

In this study, toxicity was investigated using the sixth cleavage (64-cell) stage of zebrafish embryos exposed to designed concentrations of RR 239. The results revealed that the effects of RR 239 on embryonic survival and hatching rates were concentration-dependent with an $LC_{50}$ value of 1,579 ± 25 $mg.L^{-1}$ and an $EC_{50}$ value of 1,536 ± 36 $mg.L^{-1}$, respectively, for 96 hr of exposure. The $LC_{50}$ value indicated that RR 239 is a nontoxic chemical for aquatic organisms based on the USEPA classification, and the use of RR 239 does not pose a potential environmental risk. However, RR 239 concentrations higher than 500 $mg.L^{-1}$ could reduce the embryonic survival and hatching rates and cause various morphological deformities. Survival exhibited a significant relationship to the concentration, hatching and yolk sac edema based on a cuboidal equation. Hatching influenced the survival of RR 239-exposed embryos more than either the concentration or yolk sac edema as determined by multiple linear regression. Therefore, in order to avoid the environmental risk and to protect aquatic organisms, the loading of wastewater containing RR 239 discharged by the textile industry into natural water sources should be monitored. Moreover, the effects of RR 239 on aquatic organisms living in water bodies that directly receive wastewater discharged by the textile industry should be investigated further.

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