Developmental Toxicity Study of Batik Dye using a Zebrafish Model

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ABSTRACT

The batik industry is a significant contributor to Malaysia's economy and has a long cultural history. However, improper management of batik dye wastewater poses serious environmental and public health concerns. Reactive dyes in wastewater are particularly hazardous, as they can cause haemorrhage, skin ulceration, nausea, severe dermal irritation, and dermatitis in humans. Many reactive dyes are also carcinogenic and toxic to both humans and aquatic organisms. The present study investigated the developmental toxicity of Remazol Black B dye using zebrafish (*Danio rerio*) embryos and larvae. Newly fertilised zebrafish eggs were exposed to different concentrations of the dye solution (control, 1.56 mg/L, 3.12 mg/L, 6.25 mg/L, 12.5 mg/L, and 25 mg/L) until 96 hours post-fertilisation (hpf). Developmental endpoints, including egg coagulation, tail detachment, somite formation, heartbeat presence, mortality rate, hatching success, and non-lethal malformations (skeletal deformities, swim bladder defects, and yolk sac oedema), were systematically evaluated at 8, 12, 24, 48, 60, 72, and 96 hpf. The results demonstrated significant concentration-dependent developmental alterations in zebrafish embryos exposed to Remazol Black B dye. A marked increase in mortality was observed at the highest concentration compared with the control samples. While the tested concentrations were determined to be non-toxic to humans, the findings suggest potential ecological risks, particularly to aquatic organisms, if dye effluents are discharged without adequate treatment.

Keywords: aquatic, batik dye, development, toxicity, zebrafish

1.0 INTRODUCTION

The batik industry is an important contributor to Malaysia's economy and carries a long and rich history. Historically, batik production in Malaysia can be traced back to the 15th century, although it was only during the 1970s that batik began to be widely applied and recognised in the country. Batik refers to decorative or patterned designs on a fabric or other materials, often incorporating a variety of colours and motifs. More specifically, it is defined as the process of dyeing fabric using a resist technique that prevents certain areas of the cloth from absorbing colour. The term *batik* is derived from the Javanese word *tik*, meaning "to drip" or "to make dots," while *ambatik* means "to draw, write, paint, or drip" [1].

Batik has been formally classified as a creative industry within the cultural arts sector. In recognition of its cultural significance, UNESCO has designated batik as part of the *Intangible Cultural Heritage of Humanity*, acknowledging it as a tradition and living expression inherited from ancestors to be passed on to future generations [2]. Beyond Malaysia, batik is also produced in countries such as the Philippines, Thailand, and Indonesia. In recent decades, batik makers have introduced innovative designs such as abstract patterns, stripes, and contemporary styles; expanding its applications to modern fashion and other products [3]. Today, Malaysian batik is highly valued both domestically and internationally [4].

Over time, various batik techniques have been developed, including hand-drawn batik, block batik, and screen batik, all of which are based on three fundamental processes: drawing, colouring, and finishing. Regardless of the technique, the finishing stage generally involves fixing, dewaxing, washing, and drying the fabric [2]. To enhance the distinctiveness of Malaysian batik, new techniques such as overlapping, crack, silica, discharge, and scratch methods have been introduced. These innovations enrich the aesthetic value of batik products by producing more vibrant colours and intricate designs [5].

Despite the cultural and economic importance of batik, the industry can pose environmental challenges if wastewater is not managed properly. Dyes released into wastewater have harmful effects on both humans and the environment. Large-scale batik producers may use advanced technologies to filter dyes before discharge, but in many cases, untreated wastewater is aften discharged directly into rivers and streams. Small-scale producers, particularly those located in rural

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areas, often lack the proper equipment to remove dyes from wastewater, exacerbating the problem.

Dyes are coloured substances capable of binding to substrates, typically applied in aqueous solutions with auxiliary chemicals to improve fixation. Most dyes are synthetic in origin and contain complex aromatic molecular structures, making them highly stable and resistant to biodegradation. Currently, the production of global synthetic dyes has escalated, and over 10,000 dyes are now commercially available [6]. Dyes are generally classified into three categories: anionic (acid and reactive dyes), cationic (basic dyes), and non-ionic (disperse dyes) [7]. Among these, reactive dyes are among the most widely used due to their favourable characteristics. First commercialized in 1956 following their invention by Rattee and Stephens at Imperial Chemical Industries (ICI), United Kingdom, reactive dyes rapidly gained popularity across the global textile industry [8]. Reactive dyes typically consist of azo-based chromophores coupled with various reactive groups [9]. These highly coloured organic molecules form covalent bonds with fibres during the dyeing process, resulting in strong attachment between the dye and the substrate.

Reactive dyes can be further classified according to their chemical constitution, including monochlorotriazine (MCT) dyes, vinyl sulphone (VS) dyes, heterocyclic halogen-containing (HHC) dyes, and mixed types such as MCT-VS [10]. Their covalent bonding mechanism distinguishes them from other colorants, which rely mainly on physical absorption or mechanical retention. While reactive dyes exhibit strong fixation and excellent wash and light fastness [12], they also present several drawbacks. Hydrolysis of reactive dyes during printing, caused by reaction with hydroxide ions in the paste, can reduce efficiency. Furthermore, exposure to reactive dyes is associated with potential health hazards. Reports have documented cases of occupational asthma and rhinitis linked to reactive dye exposure [13]. Positive skin prick tests and radioallergosorbent tests (RAST) have confirmed that dye molecules in the air may act as haptens, triggering production of reactive dye-specific immunoglobulin E (IgE) antibodies [14]. In most cases, allergic responses, such as rhinitis and asthma, developed within a year of exposure. Other reported conditions include contact dermatitis and urticaria. Workers handling small quantities of dyes (e.g., in weighing and mixing) are typically at higher risk than dyers [15].

Indirect impacts occur when dyes contaminate water sources, soil, and food chains. Consuming water or food contaminated with toxic dyes can lead to bioaccumulation, resulting in long-term health problems such as endocrine disruption, carcinogenesis [16]. Aquatic species across diverse taxonomic groups are also significantly impacted by industrial effluent discharge [17]. Invertebrates, including crustaceans (shrimp and crabs) [18], as well as molluscs (clams [19] and mussels [20]), exhibit physiological stress and decreased survival rates due to the harmful effect of dyes.

Zebrafish (*Danio rerio*) are widely used in developmental biology, toxicology, and transgenic research due to their favourable characteristics [16]. In comparison to other fish species, zebrafish offer extensive morphological, biochemical, and physiological data across embryonic, juvenile, and adult stages. Notably, zebrafish embryos are transparent, enabling researchers to directly observe internal development, including blood vessel formation, under a simple low-power microscope [21].

The zebrafish genome comprises approximately 1.5 billion base pairs and encodes 26,247 protein-coding genes. Remarkably, zebrafish share about 70% of their genes with humans, making them a relevant model for biomedical research [22] and facilitating studies on biological development, genetics, and disease mechanisms [23]. In contrast, rodent models such as mice are more costly, time-consuming, and subject to stricter ethical regulations. Zebrafish also offer logistical advantages: they produce hundreds of offspring weekly, facilitating large-scale studies, and their small size allows for high-throughput toxicity assays. This has enabled comprehensive investigations into the teratogenic effects (including behavioural and morphological changes), as well as the genotoxic, mutagenic, neurogenic, and cardiogenic impacts of pollutants present in aquatic environments [24]. Developmental toxicology studies in zebrafish embryos enable researchers to evaluate endpoints such as pericardial oedema, skeletal malformations, and other abnormalities [25].

Developmental toxicity refers to structural, functional, or behavioural changes that interfere with normal growth, differentiation, or development due to environmental exposure. In humans, numerous cases of teratogenic effects and birth defects caused by toxic agents have been reported [26]. Developmental toxicants may act at different stages, including: (i) fertilization and cleavage, where exposure may prevent implantation and cause embryonic lethality; (ii) implantation to gastrulation, the most sensitive stage, where organogenesis is initiated; (iii) organogenesis, where limb, organ, and body system formation occurs, and teratogens may induce malformations; (iv) morphogenesis, involving physiological maturation until birth; and (v) postnatal development until puberty, where environmental toxicants may continue to exert effects [27–30]. The developmental stages of zebrafish, from embryo to adulthood in relation to hours post-fertilization (hpf), are crucial for evaluating compound-specific toxicity. Sensitivity to toxicants varies across these developmental periods, with certain compounds exerting pronounced effects at specific stages [31-33].

The discharge of synthetic dyes, including batik dyes, into aquatic ecosystems poses a serious threat to biodiversity and ecological balance. Due to their persistence and bioaccumulative properties, these dyes degrade water quality by reducing light penetration and dissolved oxygen levels, thereby inhibiting photosynthesis in aquatic plants and algae. Such disruptions cascade through the food web, adversely impacting primary producers, invertebrates, and higher trophic organisms such as fish and amphibians. Furthermore, many dyes and their degradation products possess toxic, mutagenic, or carcinogenic properties that impair the reproduction, growth, and survival of aquatic fauna. Collectively, these impacts contribute to biodiversity loss and destabilisation of ecosystem functions, reinforcing the critical need for toxicity evaluations of dyes using sensitive biological models, such as zebrafish. This study aimed to evaluate the developmental toxicity of the batik dye Remazol Black B using zebrafish as a model organism. Zebrafish were selected due to their genetic and physiological similarities to humans, rapid reproduction, and transparent embryonic development. By exposing zebrafish embryos to varying concentrations of dye solution, we sought to determine dose-dependent developmental effects and establish safe thresholds for dye presence in aquatic environments. The findings from this study will provide valuable insights into the ecological and health risks posed by batik dye wastewater.

2.0 MATERIALS AND METHODS

2.1 Materials

Diamira Black B, commonly known as Remazol Black B dye powder, was used as the test sample. A total of 100 mg of pure Remazol Black B dye was purchased from Sigma-Aldrich (CAS No. 17095-24-8; molecular weight: 991.79 g/mol; molecular formula: $C_{26}H_{21}N_5Na_4O_{19}S_6$). The dye was stored in a sealed container to prevent contamination prior to use.

Adult zebrafish were obtained from a commercial supplier, and several criteria must be followed to select good adult zebrafish. The adult zebrafish should between 4 and12 months old and free from any infection or disease. A 2:1 male-to-female ratio was used, with twice as many males as females. There are several differences between male and female zebrafish that can be observed based on their appearance. Male zebrafish tends to be slender, while female zebrafish appears more rounded and larger than male. In addition, males often have brighter, more luminous stripes than females. Similar to other animal species, usually male zebrafish tends to chase female zebrafish before they breed in the morning.

Adult zebrafish was kept in a medium-size aquarium tank with high flow through water system. The water system was maintained based on the OECD guidelines, and it must be free from chlorine and other additives. Tap water was used in the tank and natural water dechlorinator to remove chlorine from the water. The water was left for two days before putting the zebrafish into the tank. The water was changed every two weeks to maintain its quality and cleanliness. The pH of the water was maintained within the range of 6.8 to 8.4 measured using pH paper, and the oxygen always kept above 80%. A water filter and air stone pump were used to ensure circulation and aeration. The temperature was within 26±1°C during the whole test and checked every two to three days.

2.2 Sample Preparation

2.2.1 Egg Production

Prior to breeding, adult zebrafish were separated for two weeks to optimise spawning readiness. A 6 L breeding tank was prepared and filled with dechlorinated tap water under the same conditions as the holding tanks. A custom breeding box, modified with a mesh net at the top, was placed at the bottom of the tank to prevent adults from consuming the fertilised eggs. The box was weighted down with stones to prevent floating. Artificial decorations, such as plastic plants, were included to provide visual cues and stimulate mating behaviour.

For each breeding trial, six males and three females were selected from the holding tanks one day prior to spawning and transferred into the breeding tank. A divider was used to separate males and females overnight. On the following morning, immediately before the onset of the light cycle, the divider was removed to allow mating. Breeding was typically observed within 30–60 minutes, as zebrafish are most active in the early morning. To maximise egg yield, the largest females were preferentially selected.

After spawning, the breeding box was removed, and fertilised eggs were collected using a glass pipette. Eggs were transferred into Petri dishes and rinsed two to three times with deionised water to remove debris and unfertilised material before experimental exposure.

2.2.2 Concentrations of Remazol Black B Dye Solution

For the developmental toxicity assay, five different concentrations of Remazol Black B test solution were prepared: 25 mg/L, 12.5 mg/L, 6.0 mg/L, 3.0 mg/L, and 1.5 mg/L. Each test solution was maintained in 10 mL of E3 embryo medium and placed in 35-mm plastic Petri dishes.

The E3 embryo medium was prepared according to standard zebrafish protocols and consisted of 5.0 mM sodium chloride (NaCl), 0.17 mM potassium chloride (KCl), 0.33 mM calcium chloride ($CaCl_2$), and 0.33 mM magnesium sulphate ($MgSO_4$). This medium provides the essential ionic

composition and nutrients required for normal embryonic development.

2.3 Embryo Exposure

Fertilised eggs were separated from unfertilised ones using a glass dropper under a stereomicroscope (Leica MZ16, Leica Microsystems) connected to a digital capture device (Leica MC170 HD) and monitor. Acute toxicity testing of Remazol Black B dye was conducted in accordance with OECD test guidelines, using a static exposure method for up to 96 hours post-fertilisation (hpf).

For each concentration, 30 fertilised embryos (in groups of five per replicate) were exposed to 1.5 mL of test solution. Five treatment concentrations were used (1.5, 3.0, 6.0, 12.5, and 25 mg/L), while embryos maintained in E3 medium served as the control group. Embryo mortality was recorded at 8, 12, 24, 48, 60, 72, and 96 hpf, with dead embryos removed at each observation point. In addition, developmental malformations of the embryos, such as coagulation, irregularities in somite formation, non-detachment of the tail as well as lack of heartbeat were observed and recorded for each observation time. A stereomicroscope was used to observe the developmental changes of the embryo. At the end of experiment, the developmental changes of the embryo and larvae zebrafish were compared between each concentration and the control sample.

2.4 Statistical Analysis

One-way ANOVA followed by Dunnett's comparison test was performed, using Minitab® software (Minitab LLC, State College, PA, USA). Each experimental group was compared with its corresponding control. Statistical significance was accepted when the probability of the result assuming the null hypothesis (p) was less than 0.05.

3.0 RESULTS AND DISCUSSION

Remazol Black B is one of the reactive dyes that is commonly used in textile processes. The colour, texture, and quality of the dye make it well-suited for fabric dyeing. However, textile dye effluent can cause serious harm to the environment and aquatic ecosystems. Previous research on Remazol Black B dye has only focused on the removal of the dye from wastewater products [34]. However, no studies have reported the impact of Remazol Black B on the early stages of zebrafish development.

In the present study, zebrafish were used to assess the effects of this dye during embryonic development up until 96 hpf. Observations were limited to 96 hpf because rapid embryonic development occurs between 24 and 96 hpf. Several lethal toxicological endpoints were observed, including coagulation of eggs, failure of tail detachment, lack of somite formation, and absence of heartbeat. Embryos were considered dead if one of the lethal endpoints was recorded as positive [35].

The addition of sublethal endpoints, such as the formation of yolk sac oedema, gas bladder defects, skeletal deformities, and hatching rate in the ecotoxicity tests, was significant because such effects may occur in non-target organisms, with significant impacts on populations or ecosystems [36]. Fig. 1-7 show the developmental progression of embryos from 8 hpf until 96 hpf.

3.1 Developmental Effects on Zebrafish Embryos

In this study, early developmental abnormalities were observed when the embryos reached 12 hpf. The highest concentration (25.0 mg/L) showed the most significant changes when compared with the control and other concentrations of the Remazol Black B solution. Formation of the first somite, optic vesicle, and Kupffer's vesicle was observed in the control samples at this time. Malformations in the structure of embryos were observed at the highest concentration. This may have resulted from absorption of the dye solution into the outermost membrane of the embryos, which interfered with normal development and caused structural deformities [37].

Further developmental changes of embryos were observed at 48 hpf. At this stage, several features appeared, such as more pointed pectoral fins, dorsal and ventral stripes meeting at the tail, initiation of olfactory cilia beating, circulation in blood vessels, and other morphological formations [38]. Embryos exposed to the highest test concentration exhibited several developmental defects, including absence of heartbeat and structural deformation. According to OECD guidelines, embryos are considered dead if no heartbeat is detected.

Fig. 8 shows embryonic development at 12 hpf. Formation of somites and optic vesicles can be clearly observed in the control embryos (Fig. 8a). In contrast, at 25 mg/L of test solution (Fig. 8b), no optic vesicle formation was observed, and the embryos exhibited malformations. Fig. 9 illustrates embryonic development at 48 hpf. In the control embryos (Fig. 9a), normal development was observed, including melanophore pigmentation, tapering of yolk extension, pointed pectoral fins, and formation of body stripes. In contrast, embryos exposed to 25.0 mg/L of test solution (Fig. 9b) showed no normal development, absence of heartbeat, and severe structural deformation.

3.2 Developmental Effects on Early Larvae

Development of larvae occurred after the hatching process at 48 hpf. During this period, several structures developed, including pigmentation in the retina and body, reduction in yolk sac size, and more protruding pectoral fins. Abnormal development in zebrafish larvae was observed, including the formation of yolk sac oedema at 72 hpf and uninflated gas bladders at 96 hpf. Yolk sac oedema

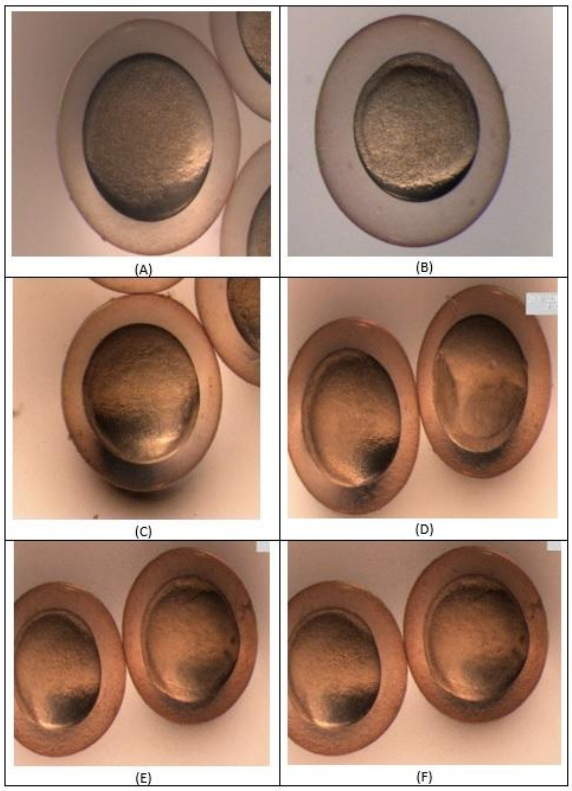

Figure 1: Development of embryo in different concentrations of test solution at 8 hpf. (A) Control (B) 1.5 mg/L (C) 3.12 mg/L (D) 6.25 mg/L (E) 12.5 mg/L (F) 25.0 mg/L.

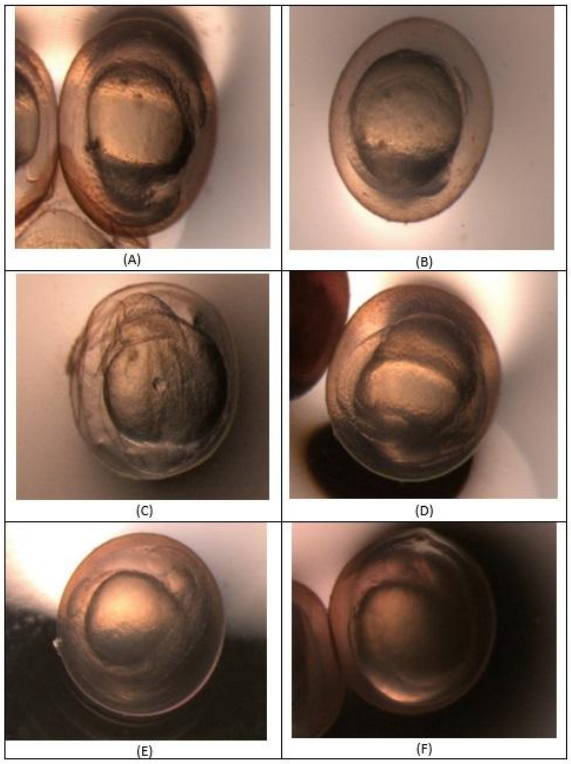

Figure 2: Development of embryo in different concentrations of test solution at 12 hpf. (A) Control (B) 1.5 mg/L (C) 3.12 mg/L (D) 6.25 mg/L (E) 12.5 mg/L (F) 25.0 mg/L.

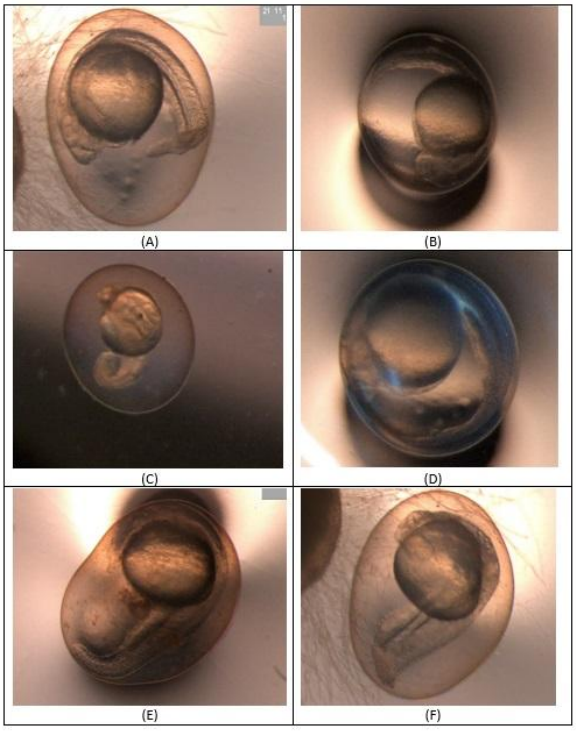

Figure 3: Development of embryo in different concentrations of test solution at 24 hpf. (A) Control (B) 1.5 mg/L (C) 3.12 mg/L (D) 6.25 mg/L (E) 12.5 mg/L (F) 25.0 mg/L.

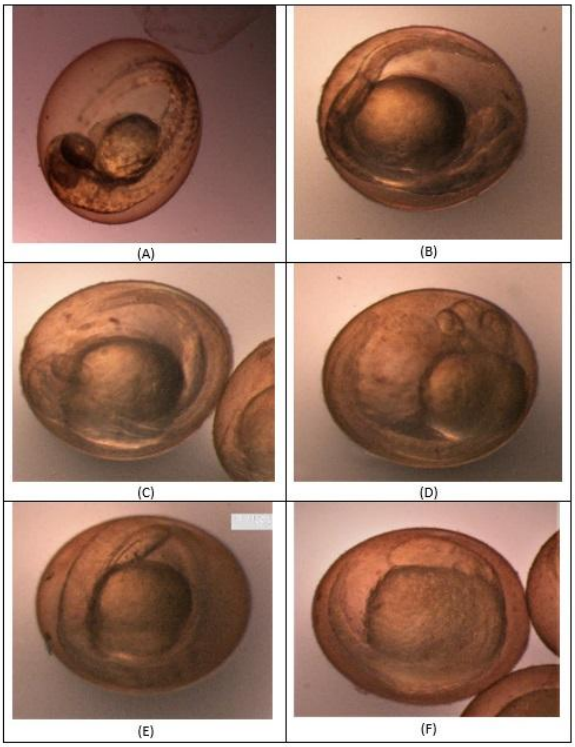

Figure 4: Development of embryo in different concentrations of test solution at 48 hpf. (A) Control (B) 1.5 mg/L (C) 3.12 mg/L (D) 6.25 mg/L (E) 12.5 mg/L (F) 25.0 mg/L.

Figure 5: Development of embryo in different concentrations of test solution at 60 hpf. (A) control (B) 1.5 mg/L (C) 3.12 mg/L (D) 6.25 mg/L (E) 12.5 mg/L (F) 25.0 mg/L.

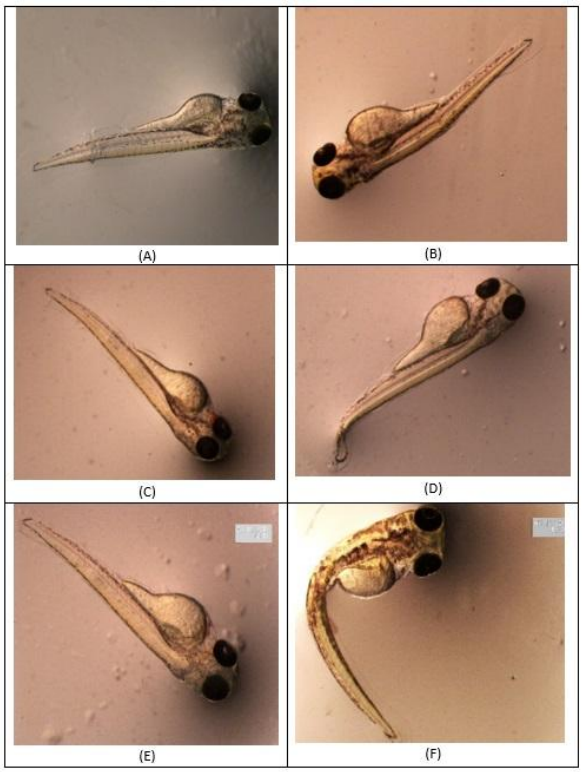

Figure 6: Development of embryo in different concentrations of test solution at 72 hpf. (A) control (B) 1.5 mg/L (C) 3.12 mg/L (D) 6.25 mg/L (E) 12.5 mg/L (F) 25.0 mg/L.

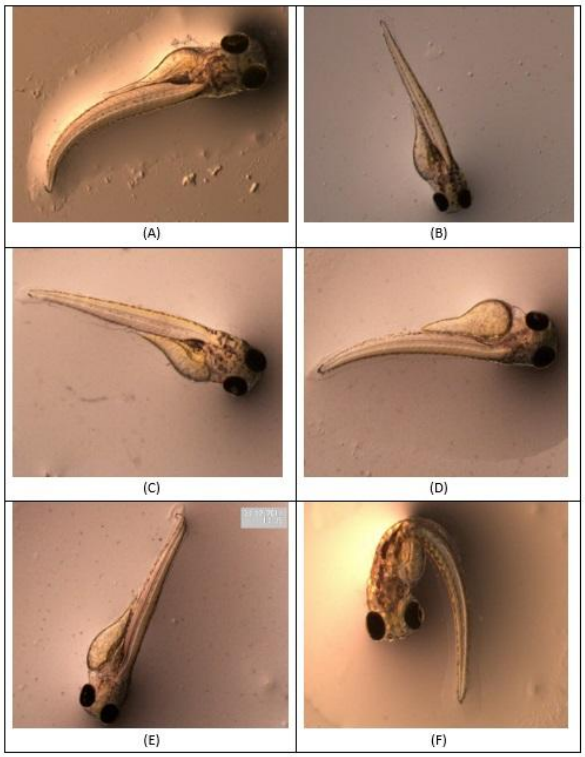

Figure 7: Development of embryo in different concentrations of test solution at 96 hpf. (A) Control (B) 1.5 mg/L (C) 3.12 mg/L (D) 6.25 mg/L (E) 12.5 mg/L (F) 25.0 mg/L.

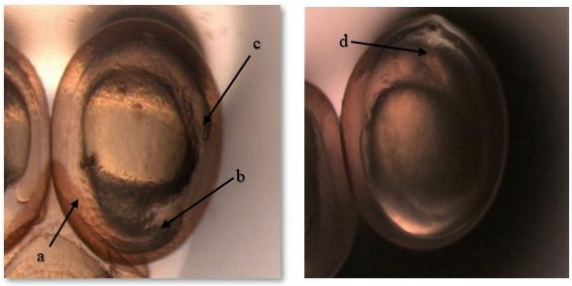

Figure 8: Development of embryo after 12 hpf between control (A) and 25 mg/L test solution (B). a: somatic formation b: optic vesicle c: Kupffer vesicle d: skeletal malformation.

was recorded at the highest test concentration (25.0 mg/L).

Fig. 10 depicts larval development at 72 hpf. A reduction in yolk sac size compared to the previous observation time was noted in the control larvae (Fig. 10a). In contrast, larvae exposed to 25.0 mg/L of the test solution (Fig. 10b) exhibited swollen and enlarged yolk sac, indicating the formation of oedema.

The yolk sac oedema formation may result from several factors. Previous studies have reported that a severe reduction in heart rate, lack of movement, and imbalances in osmotic pressure contribute to the development of oedema and body curvature. However, the mechanism by which

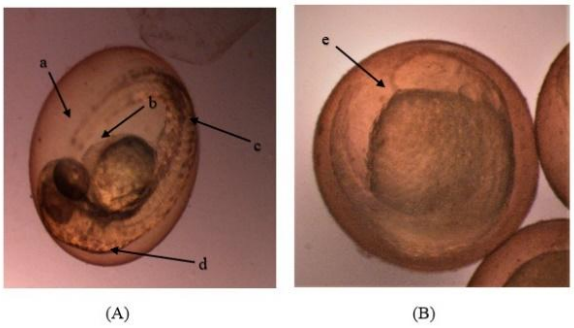

Figure 9: Development of embryo at 48 hpf between control (A) and 25.0 mg/L test solution (B). a: pectoral fin b: yolk extension c: formation of stripe d: melanophore pigment e: deformation of structure.

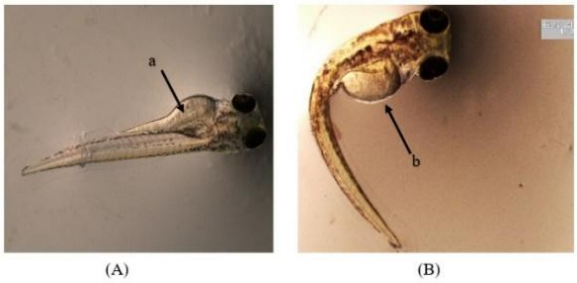

Figure 10: Development of larvae at 72 hpf between control (A) and 25.0 mg/L test solution (B). a: yolk sac b: yolk sac oedema.

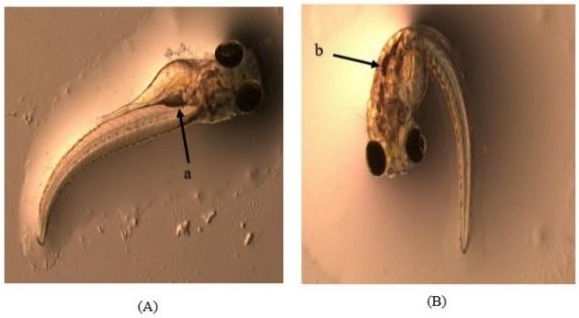

Figure 11: Development of larvae at 96 hpf between control (A) and 25.0 mg/L test solution (B). a: gall bladder b: reducing size of gall bladder.

Remazol Black B induces morphological malformations remains unclear. Yolk sac oedema is considered an important toxicological endpoint because the yolk sac provides vital nutrients for larval movement and plays a critical role in the early development of zebrafish [39].

Fig. 11 shows the development of larvae at 96 hpf. A gas bladder defect was observed in the exposed larva (Fig. 11b). The swim bladder was uninflated compared with the normal development in the control larva (Fig. 11a).

The formation of the swim bladder in zebrafish begins during the pharyngula period (24–48 hpf). However, the initial defect can only be observed at approximately 72 hpf. In the present study, the uninflated swim bladder was recorded at 96 hpf, as no observations were conducted immediately after 72 hpf. Defects in the zebrafish swim bladder disrupt essential functions such as swimming behaviour and buoyancy. These impairments negatively affect the ability of the fish to capture food and escape from predators, which may ultimately lead to injury or death [40]. Consequently, the swim bladder can be considered as a primary target organ in toxicological research, since the presence of a non-inflated swim bladder is one of the most prominent malformations observed in exposed larvae [41].

3.3 Mortality Rate

Table 1 presents the mortality rate of zebrafish embryos at each concentration and observation time. The concentrations of 12.5 mg/L and 25.0 mg/L of the test solution resulted in the highest mortality rates compared with the lower concentrations. Embryo death was first observed after 48 hpf. No mortality was recorded in the control group or at 1.56 mg/L of the test solution. Dunnett's post hoc test revealed that mortality differed significantly from 48 hpf onwards in dye-treated groups compared with the control.

In the present study, the mortality results indicate that the test solution at the highest concentration induced greater mortality compared with the lower concentrations. However, similar mortality rates were observed at concentrations of 25.0 mg/L and 12.5 mg/L. These results suggest that the tested concentrations are unlikely to pose direct harm to humans. However, they may become harmful at concentrations capable of causing complete embryo mortality [37]. Although low concentrations do not typically result in fish mortality, they may still exert toxic effects on aquatic organisms.

Table 2 shows the toxicological endpoints for zebrafish embryos exposed to different concentrations of the test solution. Lethal abnormalities, such as egg coagulation, absence of tail detachment, absence of somite formation, and lack of heartbeat were observed. Egg coagulation was observed at every observation point. Tail detachment did not occur in any exposed embryos. However, somite formation was observed in all exposed embryos. Sublethal abnormalities, such as yolk sac oedema and gas bladder defects, were observed when the larvae reached 72 hpf.

The hatching rate indicated that the hatching process continued even after 72 hpf. Under normal condition, zebrafish embryos typically hatch at approximately 48 hpf [38]. The failure of embryos to hatch might have been due to the reduction of embryonic movements caused by Remazol Black B, which disrupted the normal distribution of hatching enzymes. Consequently, the embryos could not break through the non-digestible outer layer of the egg envelope [37].

Table 1: Mortality rate of zebrafish embryo for every observation time.

Concentration	Exposure time (Post Fertilisation)					
	8hpf	24hpf	48hpf*	60hpf*	72hpf*	96hpf*
Control	X	X	X	X	X	X
1.56 mg/L	X	X	X	X	X	X
3.12 mg/L	X	X	1	X	X	X
6.25 mg/L	X	X	1	X	1	X
12.5 mg/L	X	X	1	1	X	1
25.0 mg/L	X	X	2	X	1	X

X: No mortality, *p<0.05 statistically different from the control.

Table 2: Toxicological endpoint.

Toxicological endpoint	Exposure time (post fertilisation)					
	8hpf	24hpf	48hpf	60hpf	72hpf	96hpf
Coagulation	X	X	X	X	X	X
Tail detachment						
No somite						
No heartbeat			X	X	X	X
Yolk sac oedema					X	X
Gas bladder defect					X	X
Skeletal deform					X	X
Hatching rate			X	X	X	X

X: Development

While this study provides valuable insights into the developmental toxicity of Remazol Black B dye during zebrafish embryogenesis, several limitations should be acknowledged. The present work was limited to acute exposure (≤96 hpf), focusing on the early developmental stages of zebrafish. As such, potential chronic outcomes, including long-term effects on growth, reproduction, behaviour, and transgenerational impacts, were not assessed. This limitation reduces the ability to fully understand the long-term ecological and health risks associated with Remazol Black B exposure.

The study was conducted under controlled laboratory conditions, which may not fully represent the complex interactions that occur in natural aquatic ecosystems. In the environment, dye effluents often coexist with other contaminants (e.g., heavy metals, salts, surfactants), leading to synergistic or antagonistic toxic effects that were not captured in this study.

The toxicological endpoints evaluated focused primarily on morphological and developmental changes. Although these are widely used biomarkers of toxicity, additional endpoints such as molecular, biochemical, or genomic alterations could provide deeper mechanistic insights into the dye's mode of action.

The exposure concentrations used were also based on controlled dosing regimens and may not directly reflect the fluctuating concentrations typically found in industrial effluents. Real-world conditions, such as continuous or repeated low-dose exposure, could produce different toxicity patterns from those observed under the current experimental setup.

4.0 CONCLUSION

The present study was conducted to investigate the developmental toxicity of Remazol Black B dye in zebrafish embryos. The results demonstrated a significant difference in developmental outcomes in zebrafish embryos exposed to different concentrations of Remazol Black B dye solution. Several abnormalities were observed, such as the formation of yolk sac oedema, uninflated swim bladder, and other developmental abnormalities. A significant difference in mortality rate was also recorded between embryos exposed to the highest concentration and the control group. Although the tested concentrations are considered non-toxic to humans, they may pose hazardous effects to other organisms, particularly aquatic species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

[1] Nordin R, Abu Bakar SS. Malaysian batik industry: Protecting local batik design by copyright and industrial design laws. International Journal of Business and Society. 2012;13:117–132.

[2] Akhir NHM, Ismail NW, Said R, Kaliappan SR. Creative craft: the uniqueness and potential of the Malaysian batik industry. International Journal of Interactive Digital Media. 2016;4:10-13.

[3] Haake A. The role of symmetry in Javanese batik patterns. Computers and Mathematics with Applications, Symmetry 2. 1989;17:815–826. doi:10.1016/0898-1221(89)90262-9.

[4] Akhir NHM, Ismail NW, Said R. Traditional craftsmanship: the origin, culture, and challenges of batik industry in Malaysia, in: Islamic perspectives relating to business, arts, culture and communication. Springer, pp. 229–237. 2015.

[5] Pancapalaga W, Bintoro P, Pramono YB, Triatmojo S. The evaluation of dyeing leather using batik method. International Journal of Applied Science and Technology. 2014;4(2):236-242.

[6] Ben SH, Chenari BA, Pourhassan Z, Alenezi FN, Silini A, Cherif- Silini H, et al. Diversity of synthetic dyes from textile industries, discharge impacts and treatment methods. Applied Sciences. 2021;11:6255. doi:10.3390/app11146255.

[7] Eren Z, Acar FN. Adsorption of Reactive Black 5 from an aqueous solution: equilibrium and kinetic studies. Desalination. 2006;194:1–10. doi:10. 1016/j.desal.2005.
10.022.

[8] Sawada K, Ueda M. Adsorption and fixation of a reactive dye on cotton in non-aqueous systems. Coloration Technology. 2003;119(3):182-186. doi:10.1111/j.1478-4408.2003.tb00170.x.

[9] Taylor JA. Recent developments in reactive dyes. Review of Progress in Coloration and Related Topics. 2000;30:93–108. doi:10.1111/j.1478-4408.2000.tb03785.x.

[10] Chudgar RJ. Azo dyes. Kirk-Othmer Encyclopedia of Chemical Technology. 1978;2:868–910. doi:10.1002/0471238961.01261503082104.a01.

[11] Renfrew AHM, Taylor JA. Cellulose reactive dyes: recent developments and trends. Review of Progress in Coloration and Related Topics. 1990;20;1–9. doi:10.1111/j.1478-4408.1990. tb00068.x.

[12] Glover B, Hughes JA. The physical and chemical properties of reactive dyes and their effect on printing behaviour. Journal of the Society of Dyers and Colourist. 1971;87:371–379. doi:10.1111/j. 1478-4408.1971.tb02977.x.

[13] Docker A, Wattie JM, Topping MD, Luczynska CM, Taylor AJN, Pickering CA, et al. Clinical and immunological investigations of respiratory disease in workers using reactive dyes. Occupational and Environmental Medicine. 1987:44:534–541. doi: 10.1136/oem.44.8.534 .

[14] Nilsson R, Nordlinder R, Wass U, Meding B, Belin L. Asthma, rhinitis, and dermatitis in workers exposed to reactive dyes. British Journal of Industrial Medicine. 1993;50:65–70. doi: 10.1136/oem.50.1.65.

[15] Morikawa Y, Shiomi K, Ishihara Y, Matsuura N. Triple primary cancers involving kidney, urinary bladder, and liver in a dye worker. American Journal of Industrial Medicine. 1997:31:44–49. doi:10.1002/(sici)1097-0274(199701)31:1<44:: aid-ajim7>3.0.co;2-x.

[16] Dutta S, Adhikary S, Bhattacharya S, Roy D, Chatterjee S, Chakraborty A, et al. Contamination of textile dyes in aquatic environment: adverse impacts on aquatic ecosystem and human health, and its management using bioremediation. Journal of Environmental Management. 2014;353:120103. doi:10.1016/j.jenvman. 2024.120103.

[17] Jahan S, Singh A. Causes and impact of industrial effluents on receiving water bodies: a review. Malaysian Journal of Science and Advanced Technology. 2023:111–121. doi:10.56532/mjsat.v3i2.144.

[18] Nerdy N, Lestari P, Simorangkir D, Aulianshah V, Yusuf F, Bakri TK. Comparison of chitosan from crab shell waste and shrimp shell waste as natural adsorbent against heavy metals and dyes. International Journal of Applied Pharmaceutics. 2022:181–185. doi:10.22159 /IJAP.2022V14I2.43560.

[19] Qu T, Yao X, Owens G, Gao L, Zhang H. A sustainable natural clam shell derived photocatalyst for the effective adsorption and photodegradation of organic dyes. Scientific Reports. 2022;12:2988. doi:10.1038/s41598-022-06981-3.

[20] Saha TK, Rafsan J, Yeasmine S, Mahmud Y, Moniruzzaman M, Hossain Z. Impacts of freshwater mussels on planktonic communities and water quality. Heliyon. 2023;9:e15372. doi10.1016/j.heliyon.2023. e15372.

[21] Segner H. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. Comparative Biochemistry and Physiology. Part C Toxicology and Pharmacology. 2009:149:187–195. doi: 10.1016/j.cbpc.2008.10.099.

[22] Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. Toxicological Sciences. 2005:86:6–19. doi:10.1093/toxsci/kfi110.

[23] Ferreira IJ, Meneses L, Paiva A, Diniz M, Duarte ARC. Assessment of deep eutectic solvents toxicity in zebrafish (*Danio rerio*). Chemosphere. 2022;299:134415. doi: 10.1016/j.chemosphere.2022.134415.

[24] Anifowoshe AT, Roy D, Dutta S, Nongthomba U. Evaluation of cytogenotoxic potential and embryotoxicity of KRS-Cauvery River water in zebrafish (*Danio rerio*). Ecotoxicology and Environmental Safety. 2022:233:113320. doi:10.1016/j.ecoenv. 2022.113320.

[25] Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environmental Science and Pollution Research. 2008;15:394–404. doi:10.1007/s11356-008-0018-z.

[26] Sipes NS, Padilla S, Knudsen TB. Zebrafish—As an integrative model for twenty-first century toxicity testing. Birth Defects Research Part C: Embryo Today: Review. 2011;93:256–267. doi:10.1002/bdrc.20214.

[27] Kim JH, Scialli AR. Thalidomide: the tragedy of birth defects and the effective treatment of disease. Toxicological Sciences. 2011;122:1–6. doi:10.1093/toxsci/kfr088.

[28] Rutledge JC. Developmental toxicity induced during early stages of mammalian embryogenesis. Mutation Research. 1997;396:113–127. doi:10.1016/s0027-5107(97)00178-4.

[29] Makri A, Goveia M, Balbus J, Parkin R. Children's susceptibility to chemicals: a review by developmental stage. Journal of Toxicology and Environmental Health Part B. 2004;7:417–435. doi:10.1080/10937400490512465.

[30] Grandjean P, Landrigan P. Developmental neurotoxicity of industrial chemicals. The Lancet. 2006;368:2167–2178. doi:10.1016/S0140-6736(06)69665-7.

[31] Saputra F, Uapipatanakul B, Lee J-S, Hung S-M, Huang J-C, Pang Y-C, et al. Co-treatment of copper oxide nanoparticle and carbofuran enhances cardiotoxicity in zebrafish embryos. International Journal of Molecular Sciences. 2021:22:8259. doi:10.3390/ijms22158259.

[32] Chen T, Chen H, Wang A, Yao W, Xu Z, Wang B, et al. Methyl parathion exposure induces development toxicity and cardiotoxicity in zebrafish embryos. Toxics. 2023;11:84. doi:10.3390/toxics11010084.

[33] Duan Z, Wang J, Zhang H, Wang Y, Chen Y, Cong J, et al. Elevated temperature decreases cardiovascular toxicity of nanoplastics but adds to their lethality: a case study during zebrafish (Danio rerio) development. Journal of Hazardous Materials. 2023;458:131679. doi:10.1016/j.jhazmat.2023.131679.

[34] Zuwang W. Recent developments of reactive dyes and reactive dyeing of silk. Review of Progress in Coloration and Related Topics. 1998;28:32–38. doi:10.1111/j.1478-4408.1998.tb00117.x.

[35] OECD Guidelines for the Testing of Chemicals. OECD, OECD Publishing, Paris, France. 2001.

[36] de Oliveira GAR, de Lapuente J, Teixidó E, Porredón C, Borràs M, de Oliveira DP. Textile dyes induce toxicity on zebrafish early life stages. Environmental Toxicology and Chemistry. 2016;35:429–434. doi:10.1002/etc.3202. Epub 2015 Dec 17.

[37] Shen B, Liu H-C, Ou W-B, Eilers G, Zhou S-M, Meng F-G, et al. Toxicity induced by Basic Violet 14, Direct Red 28 and Acid Red 26 in zebrafish larvae. Journal of Applied Toxicology. 2015;35:1473–1480. doi:10.1002/jat.3134. Epub 2015 Feb 27.

[38] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Developmental Dynamics. 1995;203:253–310. doi:10.1002/aja.1002030302.

[39] Li J, Liang Y, Zhang X, Lu J, Zhang J, Ruan T, et al. Impaired gas bladder inflation in zebrafish exposed to a novel heterocyclic brominated flame retardant tris(2,3-dibromopropyl) isocyanurate. Environmental Science and Technology. 2011;45:9750–9757. doi:10.1021/es202420g.

[40] Santos D, Matos M, Coimbra AM. Developmental toxicity of endocrine disruptors in early life stages of zebrafish, a genetic

[41] and embryogenesis study. Neurotoxicology and Teratology. 2014;46:18–25. doi:10.1016/j.ntt. 2014.08.002. Epub 2014 Aug 27.

[42] Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test?. Comparative Biochemistry and Physiology C Toxicology and Pharmacology. 2009;149(2):196-209. doi:10.1016/j.cbpc.2008.11.006. Epub 2008 Dec 3.