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RESEARCH

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Sub-lethal toxicity of indigo dye (*Indigofera tinctoria*) on *Oreochromis niloticus* juveniles

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Abstract

Background Textile dyes which are eliminated as unfixed dyes through the effluent from textile industry cause deleterious effect on the fresh water fish. Thus, toxicity tests were conducted using indigo dye on *Oreochromis niloticus* juveniles (mean weight 30.00 ± 0.73 g) as a test fish under bioassay system. Varying concentration of 0.00 (control), 0.5, 1.0, 1.5, 2.0, and 2.5 mg/l containing graded levels of Indigofera were applied in experimental tanks to determine the lethal concentration. The LC_{50} of dye at 96 h was 1.3 mg/l of water. The histopathology (heart, gills, kidney and spleen) and water quality parameters (Dissolved oxygen, pH and temperature) were determined using standard methods and behavioural responses were observed.

Results Histopathology of heart, gills, kidney and spleen revealed degeneration of cells, space formation, slight cellular changes and vacuolation among the treatments especially in the higher concentration of 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l of the dye solution used except in the control. The pH and DO of control were significantly different from the experimental units while there was no significant variation in the temperature of the control and all the experimental units. Effects of indigo dye on water parameters were significant ($P < 0.05$) throughout the experiment. Behavioural responses exhibited by the experimental fish include irregular swimming, hyperventilation, rapid opercula movement, and restlessness. The dye concentration and exposure period both boosted these behaviours.

Conclusions The findings of this study indicated that indigo dye solution is toxic to fish, and that fish opercula movement and mortality were influenced by the dosage of each concentration and the duration of exposure.

Keywords Behavioural responses, Cell vacuolation, Effluents, Histopathology, Indigo dye, Mortality, Textile

Background

The textile industry has been branded as one of the biggest polluters in the world due to its extensive use of chemicals and water. A dye is typically a coloured organic substance or mixture that can be used to impart color in a somewhat permanent way to a substrate like cloth, paper, plastic, or leather (Velusamy et al. 2021). Approximately 10–15% of the colours used during the dyeing process are discharged as effluents. The majority of dyes are non-biodegradable, mutagenic, carcinogenic, and possibly hazardous to humans and other living things, particularly aquatic life (Parmar and Shah 2020; Makwana 2020). Natural dyes which can also be referred to as local dyes have been used since ancient times for colouring and printing fabrics. Among these are Indigofera which is the oldest known dye and a product of Indigofera leaves. The solution contains indigotin which always appear in deep-navy blue colour when used on white fabrics (Sabina et al. 2020).

The release of textile effluents to receiving streams may be problematic for several esthetic reasons since the effluents from the dyeing and textile industries contain chemicals with strong colours. The dye effluents may contain some substances or components that could be harmful, mutagenic, carcinogenic, or poisonous to aquatic life (Nasrin et al. 2022). Aquatic species may

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experience cramping, hypertension, occasional fever, kidney damage, and other physiological issues as a result of ingesting textile effluents through the food chain (Sabina et al. 2020). Many authors (Parmar and Shah 2020; Makwana 2020; Sabina et al. 2020; Velusamy et al. 2021; Nasrin et al. 2022) have reported the toxicities of certain dyes on fishes and it was revealed that freshwater fishes are the most widely affected aquatic organisms due to pollutants. Therefore, one of the biggest risks to people and other aquatic animals is the discharge of dyes into the environment, notably from the textile and dyeing industries.

However, certain aquatic species continue to employ specific colours as a cost-effective and efficient technique of treating parasite and fungus infestations. Fish and fish eggs have traditionally used malachite green as an antifungal agent. Additionally used to cure and prevent fish disease include acriflavine and methylene blue. All of these medications are widely promoted for ornamental (non-food) fish uses, even though none of them have been licensed for use in the production of food fish. They could be employed improperly or illegally in both domestic and international aquaculture. Other dyes are commonly sold for treating external diseases in ornamental fish and show comparable antibacterial effects. The use of any of these substances in the production of edible fish is unregulated (Velusamy et al. 2021).

Despite all these information on the effects of dyes on fish, there is paucity of information on the toxicological, behavioural and growth responses of *O. niloticus* exposed to dye effluents. Since some of these local dyes especially Indigofera are still used in some parts of Nigeria to colour fabrics, it has a potential of affecting fish production through mortality, susceptibility to disease, residual effect on the fish and health hazard on the final consumer. Therefore, the purpose of this study was to examine the effects of various indigo dye concentrations on the behaviour and histopathological reactions of the spleen, gill, heart, and kidney of *Oreochromis niloticus*. Fish embryos, larvae, and early juvenile stages are considered to be the most sensitive life-history stages to toxins, which makes them the ideal time to study how the fish react to environmental contaminants (Owolabi et al. 2021). Hence, juveniles of *O. niloticus* were used in this study.

Methods

Sample collection and pre-exposure acclimation of experimental fish

Indigo dye was collected from the indigenous women involved in cottage making in Abeokuta, Ogun State, Southwest Nigeria. Doses of indigo dye were estimated and each dose was measured by micropipette and blended with aquarium water while healthy and active specimens of *Oreochromis niloticus* juveniles were obtained as experimental fish from the Teaching and Research Farm of the Federal University of Technology Akure, Ondo State Nigeria. Fishes were acclimatized under laboratory conditions for two weeks. The fishes were fed twice a day (8:00 h and 16:00 h) with commercial floating feed (40% protein) up to satiation. The water medium was changed daily. This assisted in getting rid of waste feed, feces, and other undesirable elements. All experiments were approved by the appropriate ethical review committee of the Central Research Laboratory, Federal University of Technology Akure, Nigeria.

Experimental design

After 14 days of acclimatization, based on estimated 96-h LC_{50} (1.3 mg/L), healthy juvenile of *Oreochromis niloticus* weighing 30.00 ± 0.73 g were exposed to five sublethal concentrations of indigo dye (0.5, 1.0, 1.5, 2.0, and 2.5 mg/L) and control (0.0 mg/L) in triplicates. A total of 180 individuals were allocated to eighteen 100 L glass aquaria (10 fish per tank). The fishes were fed twice a day (8:00 h and 16:00 h) with commercial floating feed (40% protein) up to satiation (Owolabi et al. 2021). Unutilized feedstuffs and fecal wastes were cleansed by siphoning. Water was changed after 24 h with previously prepared water with appropriate indigo dye dosages. Control tanks were also maintained under the laboratory condition. The death rates and behavioural disorders were observed every 24 h. Three fishes were sampled from each tank to collect heart, gill, spleen and kidney samples for histopathological examination after the exposure period was over. Samples were immediately fixed in 10% formalin for histopathological observation later.

Determination of physico-chemical properties of water

The dissolved oxygen (DO), pH and temperature concentration were determined and their readings were recorded daily. A mercury-in-glass thermometer was used to measure the temperature; it was immersed into the experimental tank for 1 min before measurements were taken. The DO concentration was determined with a DO meter (Model HI 9828) while pH was determined using a manual pH meter Ohaus starter 3100, OHAUS Corporation, USA according to Ajibare et al. (2022).

Histopathological assessment

For histopathological studies, the tissues (gill, heart, spleen and kidney) were collected in 10% formalin. They were processed routinely for paraffin embedding and sectioned to 5 µm thickness with a microtome machine (HM 430; Thermo-Scientific). The tissue slices were arranged on slides like ribbons and stained with standard Haematoxylene-Eosin (H-E) protocol before being

mounted with DPX and a coverslip. Finally, stained slides were examined under a microscope for histopathological abnormalities in the tissues (Owolabi 2011).

Calculation of percentage mortality

The percentage mortality rate was calculated according to Owolabi (2011) as follows:

$$\%\text{Mortality} = \frac{\text{Number of dead fish}}{\text{Initial number of stocked fish}} \times 100$$

Statistical analysis

Comparison of the effects of the different concentrations of indigo dye was performed by analysis of variance (ANOVA) on IBM SPSS 20.0. The means were separated with New Duncan Multiple Range Tests at $P < 0.05$ using IBM SPSS 20.0.

Results

Water quality parameters

The physico-chemical parameters of the experimental water are presented in Table 1. The table showed slight changes in all the parameters used throughout the 96 h experiment. The three parameters that were monitored were temperature, dissolved oxygen and pH. The result showed that the pH (6.70 ± 0.27) and DO (5.80 ± 0.28 mg/l) of control were significantly different from the values observed in the experimental units while there was no significant variation in the temperature of the control and all the experimental units.

Fish behavioural response to Indigo dye

The result of this study (as presented in Table 2) revealed that the 150 fish exposed to varying concentration of dye exhibited irregular swimming, hyperventilation, rapid movement of the opercula, restlessness, etc. and it was observed that the behavioural responses were dependent on concentration and exposure duration i.e. the higher the concentration of the indigo dye the higher the level of abnormality displayed by the fishes. Table 2 further revealed that the fish exposed to concentration less than 1.5 mg/l of dye in 24 h did not exhibit any abnormal behaviour while those exposed to concentration of less than 1.0 mg/l in 48 h did not display abnormal behaviours. However, the table revealed all the treatments exhibited all the observed abnormal behaviours at 72 h and 96 h while all the fish in the control tank exhibited normal behaviours throughout the experiment. Similarly, the mortality rate of exposed fish was concentration and exposure period dependent (Table 2). The table revealed that 10%, 60% and 90% mortality was recorded for 1.5 mg/l, 2.0 mg/l and 2.5 mg/l, respectively, after 24 h. The mortality rate after 48 h also revealed that 10%, 80% and 100% mortality was recorded in the fish exposed to 1.5 mg/l, 2.0 mg/l and 2.5 mg/l. Also, after 72 h, the mortality rate was 10%, 20%, 100% and 100% for 1.0 mg/l, 1.5 mg/l, 2.0 mg/l and 2.5 mg/l, respectively, while the order recorded after 96 h was 20%, 30%, 60%, 100%

Table 1 Physico-chemical parameters of water with increase in concentration of indigo dye concentration

Parameters	Control	0.5 (mg/l)	1.0 (mg/l)	1.5 (mg/l)	2.0 (mg/l)	2.5 (mg/l)
Temp (°C)	22.78 ± 0.71^{a}	22.78 ± 0.70^{a}	22.75 ± 0.47^{a}	22.75 ± 0.69^{a}	22.75 ± 0.69^{a}	22.76 ± 0.70^{a}
pH	6.70 ± 0.27^{a}	7.71 ± 0.15^{b}	8.72 ± 0.16^{b}	7.14 ± 0.42^{b}	7.69 ± 0.22^{b}	7.30 ± 0.21^{b}
DO (mg/l)	5.80 ± 0.28^{a}	4.14 ± 0.22^{b}	4.14 ± 0.22^{b}	4.31 ± 0.27^{b}	4.51 ± 0.23^{b}	4.84 ± 0.23^{b}

Mean values in the same row with the same superscript do not differ significantly at $P > 0.05$

Table 2 Behavioural responses of *Oreochromis niloticus* juveniles exposed to different levels of Indigo dye (*Indigofera tinctoria*) in definitive test at different time intervals

	24 h						48 h						72 h						96 h					
Concentration (mg/l)	0.0	0.5	1.0	1.5	2.0	2.5	0.0	0.5	1.0	1.5	2.0	2.5	0.0	0.5	1.0	1.5	2.0	2.5	0.0	0.5	1.0	1.5	2.0	2.5
Erratic swimming	−	−	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
Loss of reflex	−	−	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
Hyperventilation	−	−	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
Changes in behaviour	−	−	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
Discolouration	−	−	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
% Mortality (%)	−	−	−	10	60	90	−	−	−	10	80	100	−	−	10	20	100	100	−	20	30	60	100	100

+, Presence of specific observation; −, absence of specific observation

Table 3 Histopathological observation in the heart, gills, kidney and spleen of *O. niloticus* juveniles exposed to varying concentration of indigo dye

Conc (mg/l)	Heart	Gills	Kidney	Spleen
0.00	Normal features were seen (Fig. 1a)	Normal features were seen (Fig. 1b)	Normal features were observed (Fig. 1c)	Normal features were observed (Fig. 1d)
0.50	Slight Cellular changes (Fig. 2a)	Slight vacuolation (Fig. 2b)	Slight cellular changes, vacuolation but dense nucleus (Fig. 2c)	Space formation and Slight Cellular changes (Fig. 2d)
1.00	Slight Cellular changes and Degeneration (Fig. 3a)	Low vacuolation with inflammation (Fig. 3b)	Space formation and Scattering of Cells (Fig. 3c)	Space formation and vacuolation (Fig. 3d)
1.50	Degeneration of Cells (Fig. 4a)	Slight degeneration, vacuolation and slight congestion of filament (Fig. 4b)	Shrinkage of nucleus and Space formation (Fig. 4c)	Dense nucleus (Fig. 4d)
2.00	Dissolution of Cells and Degeneration (Fig. 5a)	High level of degeneration and inflammation (Fig. 5b)	High dissolution of nucleus and Space formation (Fig. 5c)	High level of vacuolation and dissolution of nucleus (Fig. 5d)
2.50	Total dissolution and degeneration of cell-structure (Fig. 6a)	Extensive necrosis and total degeneration of filament (Fig. 6b)	Total dissolution of nucleus with loss of chromatin materials (Fig. 6c)	Total dissolution of nucleus with loss of chromatin materials (Fig. 6d)

and 100% for 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l and 2.5 mg/l, respectively. The table showed that there was no mortality in the control experiments throughout the study.

Histopathological observations

The histopathological observations of the heart, gills, kidney and spleen of *O. niloticus* juveniles exposed to varying concentration of indigo dye are presented in Table 3 and Figs. 1, 2, 3, 4, 5, 6. The result revealed that some alterations including vacuolation, space formation, scattering of cells, shrinkage and dissolution of nucleus as well as degeneration of cell structure were observed in the organs (heart, gills, spleen and kidney) of the fish exposed to different concentration of indigo dye solution especially at high concentration of the dye (Table 3; Figs. 1, 2, 3, 4, 5, 6).

Discussion

The discharge of indigo dye into water as revealed by the minor acidic range of the pH (5.80–7.55) recorded, may contribute to an increase in the primary productivity of water bodies. Researchers such as Odeyemi et al. (2018), Deepika and Noorjahan (2018), Velusamy et al. (2021), Alaguprathana and Poonkothai (2021) and Nasrin et al., (2022) have also reported similar effects of dyes on aquatic media and attributed the rise in loads of major ions to organic pollution. The abnormal behavior changes such as fast jerking, frequent jumping, erratic swimming, spiraling, convulsion, and a propensity to escape the experimental tanks, are consistent with those discovered by Selvaraj et al. (2015) and Kishore et al. (2022) in *Poecilia recticulata* and *Clarias batrachus*, respectively.

The result of this study also revealed that indigo dye were toxic to the experimental fish, resulting in both external and internal harm, including body darkening, copious mucous secretion (a defense mechanism), and dye deposition over the exterior (gills) and internal organs (lateral line and digestive system). This observation was in agreement with the findings of Jacquin et al. (2020), Alaguprathana and Poonkothai (2021) and Barathinivas et al. (2022) who reported that fish exposed to sub-lethal levels of pollution typically exhibit hyperactivity, changes in opercula rate, unpredictable and sudden jerky swimming movements, and other behavioural patterns, which have been shown to be sensitive indicators of physiological stress. Also, Ajibare et al (2022) suggested that increased opercular movement in fish may be a sign of stress and may lead to physiological dysfunction in the fish. This opercular movement may be brought on by a decrease in the efficiency of oxygen intake or transport. The 96 LC_{50} of indigo dye to *O. niloticus* was 1.3 ml/l. The rate at which the organisms die was also used as criterion for toxicities (dye effect). Other behaviours observed before the death of the organisms were loss of reflex, discolouration, high rate of respiration. The results of this study supported the findings of Authman and Abbas (2007) that the exposure of *Oreochromis mossambicus* to ammonia caused clinical indications such unusual movements and abnormal respiration rate that presented brain dysfunction and gill damage. Therefore, increased gill injury may be contributing to the high fish mortality rate by impairing gaseous exchange (Jacquin et al. 2020).

Findings of this study about the morphological changes in gills were consistent with several earlier similar studies (Parmar and Shah 2020; Alaguprathana and Poonkothai 2021; Kishore et al. 2022). Fish exposed to dye had gills with hyperplasia, aneurisms, raising of the gill epithelium, twisted secondary gill lamellae, and a degraded central axis (Jagruti 2015; Parmar and Shah 2020). Selvaraj et al. (2015), Makwana (2020) and Gao et al. (2021) have also documented histopathological alterations (including expansion of the primary gill bar, detachment of the secondary gill bar, and haematocyte infiltration into the lumen) in *Poecilia recticulata*, *Clarias batrachus* and *Takifugu rubripes*, respectively. However, in the presence of dye or other toxicants, epithelial lifting has been demonstrated by Parmar and Shah (2020) as a protective response to maintain the distance between secondary gill lamellae and toxicant, making secondary gill lamellae curvier and potentially affecting the structure and functions of the entire gill.

This study's histopathological findings in the kidney and gill were highly congruent with earlier studies by Athira and Jaya (2018) and Sabina et al. (2020), which established that fish gill exhibit cytoplasmic vacuolation and bunching of nuclei after exposure to textile dyeing effluent, and that the kidney experiences nucleus breakdown with loss of chromatin materials. The histopathological observation of the vital organs of fish exposed to low sub-lethal concentrations of indigo dye (as shown in Figs. 2 and 3) revealed that there was slight cellular changes (space formation and scattering of cells) low vacuolation with inflammation. However, there was extensive necrosis and total degeneration of cell /nucleus with loss of chromatin materials at very high concentration of indigo dye (Fig. 6). Similarly, Fatma and Mohamed (2009) observed intravascular hemolysis in hepatic blood vessels and hepatoportal blood vessels, as well as vacuolar degeneration in the hepatocytes, focal areas of necrosis and aggregations of inflammatory cells between the hepatocytes, dilation and thrombosis formation in central veins, dilation and congestion in blood sinusoids in the liver of *Tilapia zilli* and *Solea vulgaris* from the lake Qarun contaminated with different pollutants.

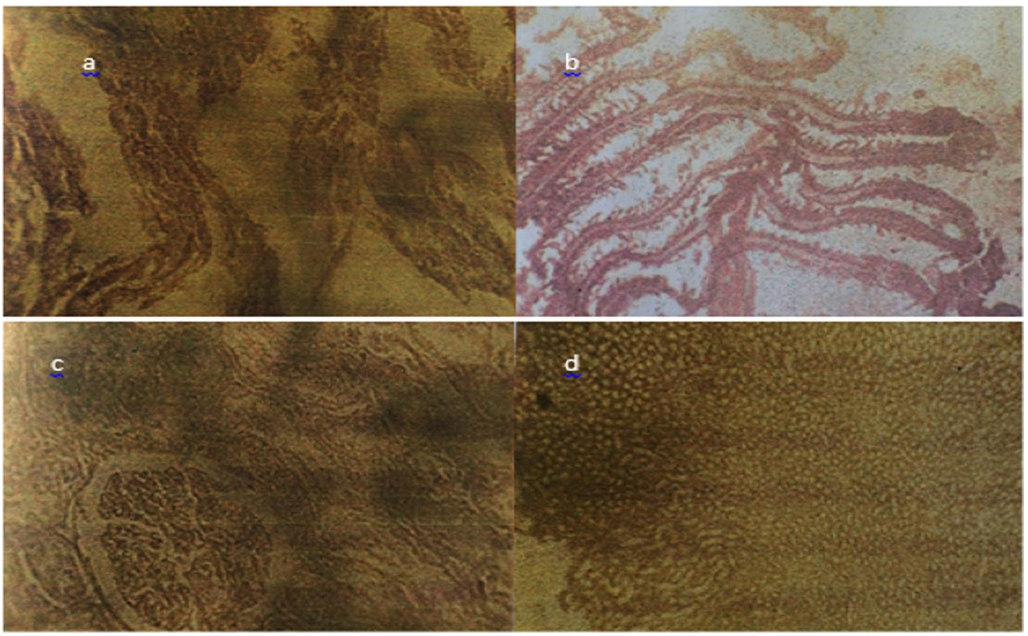

Fig. 1 Histopathological observations in the organs of *O. niloticus* in the control experiment (**a** Heart, **b** Gill, **c** Kidney, **d** Spleen)

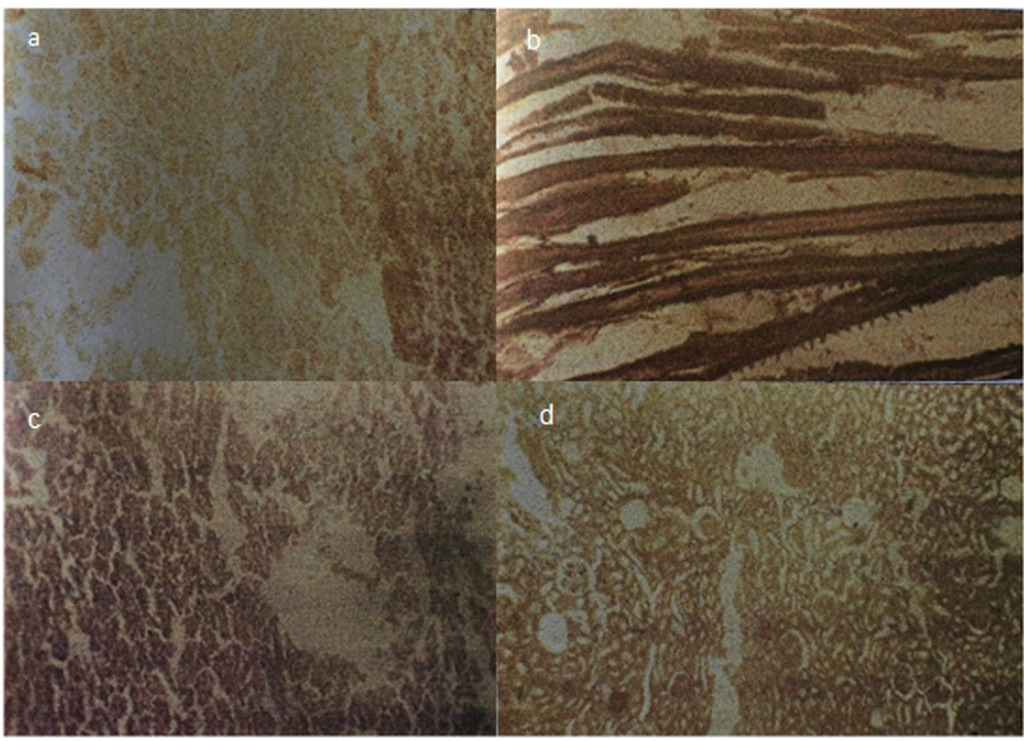

Fig. 2 Histopathological observations in the organs of *O. niloticus* exposed to 0.5 ml/L of dye (**a** Heart, **b** Gill, **c** Kidney, **d** Spleen)

Additionally, Koca et al. (2008), Kaur and Mishra (2019) documented a number of histopathological changes including inflated and ruptured parenchymal cells, loss of cord structure, vacuoles packed with cellular debris, focal necrosis, and a significant rise in kupffer cells in the liver as a result of accumulation of toxicants.

The outcome of this study was corroborated by earlier investigations on the effects of various dyes on different

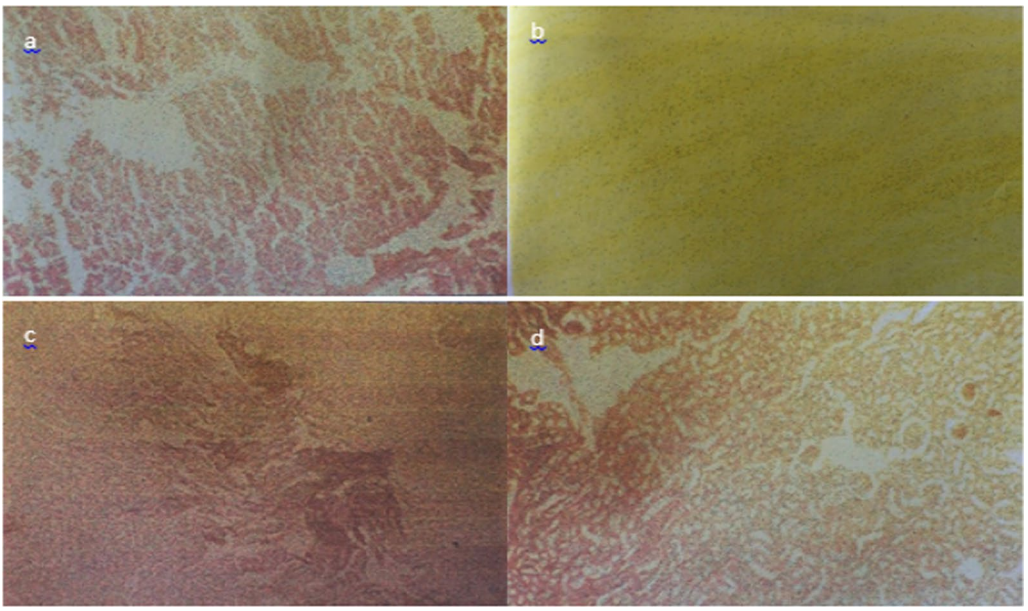

Fig. 3 Histopathological observations in the organs of *O. niloticus* exposed to 1.0 ml/L of dye (**a** Heart, **b** Gill, **c** Kidney, **d** Spleen)

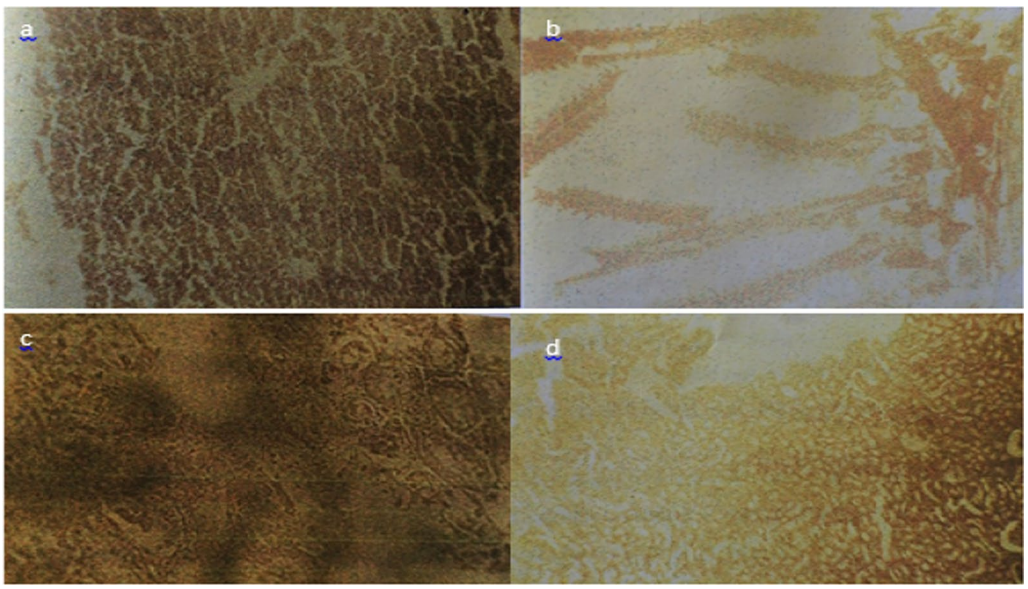

Fig. 4 Histopathological observations in the organs of *O. niloticus* exposed to 1.5 ml/L of dye (**a** Heart, **b** Gill, **c** Kidney, **d** Spleen)

species of fish (Avni and Barot 2016; Deepika and Noorjahan 2018; Parmar and Shah 2019, 2020; Velusamy et al. 2021; Alaguprathana and Poonkothai 2021). This research also supports the findings of Kirandeep et al. (2015), who demonstrated that the concentration-dependent cytogenotoxicity of the azo dye Acid Blue-113 caused harm to all examined target tissues (brain, liver, kidneys, and reproductive system) in the study of *C. punctatus*. Moreover, the study's findings on the toxicity of indigo dye are consistent with the experiment done by Al-Sabti (2000) on the genotoxic effects of textile dyes like chlorotriazine-reactive azo red on aquatic ecosystems. As a result, our work has shown that information on fish mortality and their histopathological and behavioral responses is relevant for estimating the sub-lethal toxicity of contaminants. In order to start corrective measures for ecosystem health, water managers who rely on fish survival data for wastewater discharge in water courses must also

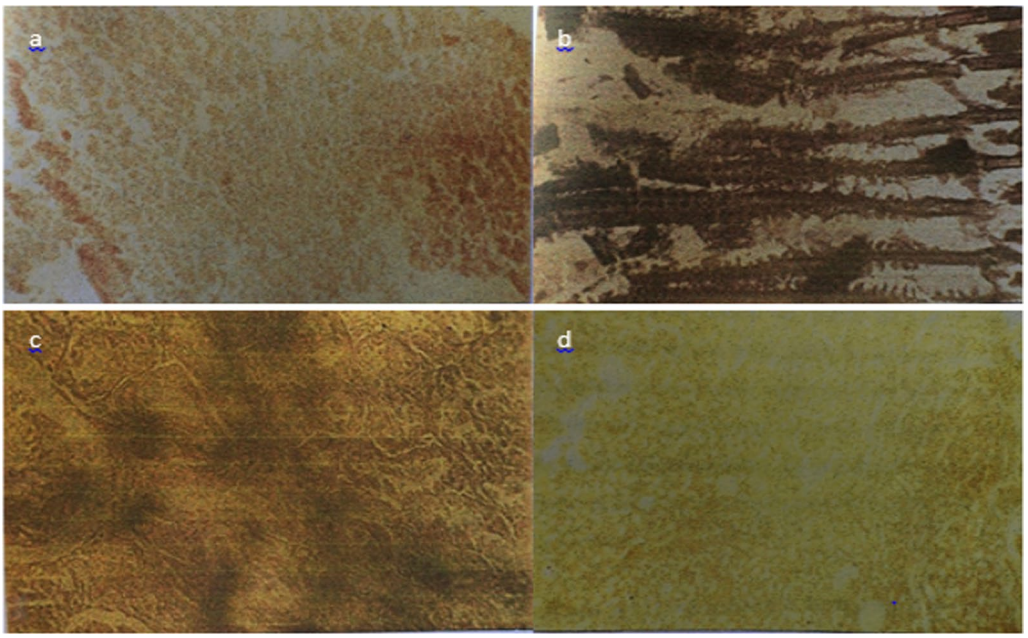

Fig. 5 Histopathological observations in the organs of *O. niloticus* exposed to 2.0 ml/L of dye (**a** Heart, **b** Gill, **c** Kidney, **d** Spleen)

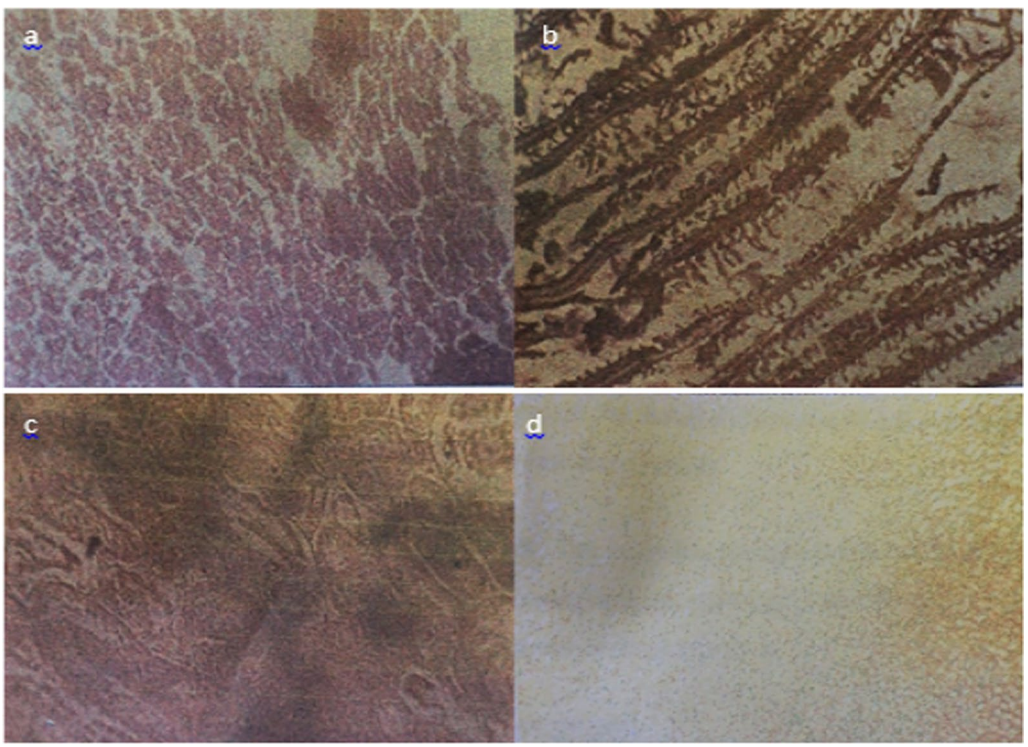

Fig. 6 Histopathological observations in the organs of *O. niloticus* exposed to 2.5 ml/L of dye (**a** Heart, **b** Gill, **c** Kidney, **d** Spleen)

analyze fish health for detecting toxicity at higher dilutions. Therefore, the government must take action to prevent the indiscriminate siting of small- and large-scale textile mills in order to protect the environment since indigo dye and textile effluents pose a possible risk to the receiving water body.

Conclusions

According to the findings of this study, *Oreochromis niloticus* exhibited morphological, behavioral, and histopathological changes at various concentrations of indigo dye. The responses of *Oreochromis niloticus* to the various concentrations of indigo dye solution showed that the dye (Indigofera) was toxic to the fish while the dosage of each concentration and the length of exposure time affected the fish opercula movement and histopathological changes such as degeneration of cells, space formation, slight cellular changes and vacuolation (as observed in the fish). Also, exposure to indigo dyes suppressed the aerobic respiration and triggered the anaerobic respiration. Therefore, in order to maintain the aquatic system, the indiscriminate use of synthetic chemical dyes should be limited and replaced with eco-friendly dyes. Moreover, proper usage, handling and disposal should be strictly considered and monitored.

Abbreviations

ANOVA	Analysis of variance
DO	Dissolved oxygen
H-E	Haematoxylene-Eosin
LC_{50}	Median lethal concentration
Temp	Temperature

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Author contributions

All of the authors worked together to complete this project. AOV and AAO conceptualised, designed and participated in the work. The protocol was written by AOV and AAO. The statistical analysis was carried out by AAO. The study's analyses and literature searches were handled by AAO. The original draft of the manuscript was written by AOV and AAO. The final manuscript was read, corrected and approved by both authors.

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Availability of data and materials

All data generated or analysed during this study are included in this article. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

The authors confirm that all experiments were carried out following the approval of the appropriate ethical review committee of the Central Research Laboratory, Federal University of Technology Akure, Nigeria (reference number not applicable) and were in accordance with both the national and international safety regulations and ethical principles for animal welfare. The authors also confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no financial or non-financial competing interests that are directly or indirectly related to this work.

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