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Toxicity of Titanium Dioxide Nanoparticles to Tadpoles of Asian Common Toad (*Duttaphrynus melanostictus*) Following Short Term and Chronic Exposures

K. M. Sudesh Ruvinda[1] · Asoka Pathiratne[1]

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Abstract
Nanotoxicity data for amphibians are limited compared to other taxonomic groups. The present study assessed toxicity of titanium dioxide nanoparticles (nanoTiO_2, anatase form, particle size < 25 nm) on tadpoles of *Duttaphrynus melanostictus* after short term and chronic exposures. Exposure to nanoTiO_2 (≤ 100 mg/L) for 96 h had no significant effect on survival but upon transfer to nanoTiO_2 free water for another 21 days, survival of the tadpoles pre-exposed to nanoTiO_2 was greatly reduced and their development was delayed. Chronic exposure to nanoTiO_2 (0.1 to 10 mg/L) for 14 days had no significant effects on acetylcholinesterase activities but induced mortalities (up to 40%) and histological alterations in the intestine and liver tissues. The results indicate that release of nanoTiO_2 to aquatic ecosystems could pose negative impacts to amphibian populations.

Keywords Nanotoxicity · NanoTiO_2 · Amphibian larvae · Histopathology · Acetylcholinesterase

In the recent decades, engineered nanomaterials especially titanium dioxide nanoparticles (nanoTiO_2) are abundantly manufactured and increasingly used in wide variety of commercial products and industrial processes (Robichaud et al. 2009; Abdel-Latifa 2020). With the extensive usage, terrestrial and aquatic ecosystems can be contaminated with such nanomaterials posing unforeseen consequences to biota. Although considerable toxicity studies have been carried out to assess the potential impact of nanoTiO_2 on different taxonomic groups, nanotoxicity data related to amphibians are limited (Menard et al. 2011; Amaral et al. 2019). Contamination of the habitats of the amphibian larvae with such nanomaterials mainly through discharge of raw sewage, wastewaters, and industrial effluents may pose potential adverse effects on amphibian populations (Amaral et al. 2019).

Duttaphrynus melanostictus (Asian common toad) which is categorized under 'least concern' in the global amphibian assessment, is a widely distributed bufonid amphibian in tropical Asia (IUCN 2004). This study aimed at assessing the potential toxicity associated with short term and chronic exposures of a range of concentrations of nanoTiO_2 on amphibian larvae using tadpoles of *D. melanostictus* as the model organism in order to generate tropical nanotoxicity data which would be useful for future ecological risk assessments. Subsequent to the short term exposure, potential recovery of the tadpoles exposed to nanoTiO_2 was assessed using survival, body size and development stage as endpoints. After the chronic exposure, acetylcholinesterase (AChE) activity of the tadpoles was evaluated as a biomarker of potential neurotoxicity. NanoTiO_2 induced alterations of AChE activities have been reported earlier in some other taxa (Guan et al. 2018; Amaral et al. 2019). In addition, histological structure of selected organs of the tadpoles was examined in order to assess structural damage.

Materials and Methods

Titanium dioxide nanoparticles (CAS number: 1317-70-0, Anatase form in crystal structure) was obtained from Sigma-Aldrich, USA. According to the manufacturer, particle size of nanoTiO_2 is < 25 nm, purity is 99.7% on trace metal basis and specific surface area is 45–55 m^2/g. NanoTiO_2

✉ Asoka Pathiratne
asoka@kln.ac.lk

[1] Department of Zoology and Environmental Management, University of Kelaniya, Kelaniya 11600, Sri Lanka

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was morphologically characterized by transmission electron microscopy (JEOL - JEM - 2100) and size distribution in solution was monitored by a dynamic light scattering analyzer (Malvern Zetasizer Nano ZS) at Sri Lanka Institute of Nanotechnology (Pvt.) Ltd., Sri Lanka. Tadpoles of *D. melanostictus* were collected from a pond in an unpolluted area in the Gampaha District, Sri Lanka. This pond was considered as an unpolluted water body. Industrial, agricultural or urban pollution sources were not found in this area. Tadpoles were brought to the laboratory in aerated pond water in polythene bags and allowed to acclimate to laboratory conditions in glass tanks with aerated aged tap water for four days under natural photoperiod (12 h light:12 h dark cycle). Tadpoles were fed daily twice with commercially available fish meal (Prima, Sri Lanka). Temperature, pH and dissolved oxygen concentration in water during acclimation period (mean ± SEM) were 27.6 ± 0.1°C, 8.15 ± 0.03 and 4.84 ± 0.03 mg/L respectively. The developmental stages of the acclimated tadpoles were examined before the exposure studies and only the free swimming stage of tadpoles at 'Gosner stage 25' (McDiarmid and Altig 1999) was used for all toxicity tests. Tadpoles used in the toxicity studies were (mean ± SEM) 7.2 ± 1.2 mg in body weight, 3.6 ± 0.5 mm in snout to vent length (SVL) and 8.6 ± 1.1 mm in snout to tail end length (STL). Toxicity tests were conducted following the OECD guidelines (2009) under static-renewal system. Stock suspensions of nanoTiO_2 were freshly prepared in deionized water and were dispersed for one hour with sonication (bath type sonicator, Sonar 203 H, 53 kHz frequency, Rocker Scientific Ltd., Taiwan) immediately prior to use. Required volumes of dispersed nanoTiO_2 stock solutions were added separately into the glass tanks each containing aged tap water and mixed thoroughly to facilitate homogenous dispersion before introducing the tadpoles. Aliquots of these exposure media were acidified to pH = 2 with concentrated nitric acid and titanium (Ti) content in each exposure medium was quantified by atomic absorption spectroscopy, graphite furnace mode (Analytik Jena: Model novAA400P). Measured concentration of Ti in the samples were within 90%–110% of the Ti content in the nominal TiO_2 suspension. Limit of quantification was 0.03 mg/L of Ti.

For short term toxicity test (96 h), acclimated tadpoles were placed in groups of 20 individuals in separate glass aquaria containing 2 L of different concentrations (5, 10, 30, 50, 100 mg/L) of nanoTiO_2. The tadpoles placed in glass tanks with only aged tap water were used as controls. Three replicate tanks were set up for each concentration of nanoTiO_2 and the controls. Each exposure medium was renewed at 48 h. The tadpoles were not fed and their mortality was recorded at 24 h intervals for 96 h. Temperature, pH and dissolved oxygen concentration in water (minimum-maximum value) during the exposure period were 27–28°C, 8.06–8.34 and 4.8–5.5 mg/L respectively. After the 96-h exposure, remaining tadpoles in each tank were transferred separately in to another set of glass tanks containing 4 L of only aged tap water for further observations on their survival, body size and development for additional 21 days. Tadpoles were fed with the fishmeal, twice a day (2% of body weight). Dead tadpoles and excess food were removed after 2 h of feeding to reduce the spoilage of the water medium. Every second day the aged tap water was renewed in each tank. The water quality parameters, temperature, pH and dissolved oxygen concentration in water (minimum-maximum value) during post exposure period were 27.1–28.5°C, 7.83–8.21 and 4.9–5.8 mg/L respectively.

For the chronic toxicity test (14 days), acclimated tadpoles were placed in glass tanks (20 individuals per tank) containing 2 L of different concentrations of nanoTiO_2 suspensions (0.1, 0.3, 1, 3, 10 mg/L) or 2 L of aged tap water (control). Triplicate tanks were set up for each concentration. The exposure media were renewed every 2 days and mortality of tadpoles was recorded daily for 14 days. Temperature, pH and dissolved oxygen concentration in water (minimum-maximum value) during exposure period were 27.0–27.5°C, 8.01–8.25 and 4.9–5.6 mg/L respectively. Subsequent to the 14 days exposure, 6 to 8 tadpoles from each tank were euthanized with buffered benzocaine hydrochloride (2 mg/L) solution, washed with distilled water immediately and stored at −40°C until processed for AChE assay. Of the remaining tadpoles, 3 tadpoles from each exposure group were sacrificed and preserved in neutral buffered formalin for histology.

For AChE assay, enzyme source was prepared by homogenizing the pooled anterior body portions of 6–8 tadpoles taken from each tank with ice cold pH 8, 0.1 M, NaH_2PO_4/K_2HPO_4 buffer (at a ratio of 10 mg of body portions in 2.00 mL of buffer) by tissue homogenizer (Ultra- Turrax T25, IKA Labortechnik, Germany). The homogenates were immediately used in the AChE enzyme assay following the method of Ellman et al. (1961) with slight modifications using a computer controlled UV-visible spectrophotometer (GBC Cintra 10e, Australila). The homogenate (0.7 mL) was added to a cuvette containing 25 µL of 0.01 M 5, 5-dithiobis-2-nitrobenzoic acid and vortexed the mixture and the absorbance at 412 nm was measured at 20 s intervals for one minute. The change of the absorbance at 412 nm was recorded again at 20 s intervals for two minutes after adding 20 µL of 0.075 M acetylthiocholine iodide in to the cuvette and mixed well with a plastic stirring rod. The assays were conducted in duplicates. The absorbance was corrected for the non-enzymatic reaction and the enzymatic reaction rate was calculated using the extinction coefficient of 5,5-dithiobis-2-nitrobenzoate ion (13,600 M/cm). Total protein contents in the homogenates were assessed following the procedure of Lowry et al. (1951) with bovine serum albumin as the

protein standard. AChE activity was expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein.

For histology, the preserved specimens were dehydrated and embedded in paraffin wax. Serial histological sections were obtained with the thickness of 5 µm using a rotary microtome (Leica® Reichert-Jung-2030) and stained with haematoxylin and eosin (Bucke 1998). The stained sections were observed under the compound light microscope to examine histological alterations (if any) induced by the nanoTiO_2 exposure in the intestinal and liver tissues of the tadpoles. Tissue sections of the tadpoles from nanoTiO_2 exposure groups and the controls (n = 3 tadpoles per group) were examined for histopathology.

Median lethal concentrations based on concentration-response relationships with respect to the survival data could not be obtained due to insufficient partial effects. Significant differences among controls and nanoTiO_2 exposed tadpoles with respect to the survival and final body lengths (SVL) data were tested by two sample t-test. The AChE activities of the tadpoles exposed to nanoTiO_2 were compared with respect to the control by Kruskal-Wallis test as normality of data was not found. The results were considered as significantly different if $p \leq 0.05$ (Zar 1998).

Results and Discussion

Nanotoxicity data for amphibians are limited compared to other taxonomic groups. Ecotoxicological studies of nanomaterials on native amphibians of different ecosystems are required for evaluating variations in species sensitivity (Amaral et al. 2019). For the first time, we have used tadpoles of *D. melanostictus* as the test model under tropical conditions to provide scientific evidence on potential toxicity of nanoTiO_2 on amphibians by combining 96 h short term exposure with post-exposure periods and 14 days chronic exposure to a range of concentrations of nanoparticles. The transmission electron microscopic images (Fig. 1) indicated that the maximum particle size of nanoTiO_2 was close to the value given by the manufacturers (< 25 nm). Size distribution was analyzed in the lowest (0.1 mg/L) and middle (1 mg/L) concentrations of nanoTiO_2 solutions used in the chronic exposure study. Particle size ranges of the suspensions of 0.1 and 1 mg/L nanoTiO_2 at 24 h were respectively from 459 nm to 615 nm and 1281 nm to 2669 nm indicating aggregations of primary nanoparticles into larger secondary particles. It can be expected that much larger aggregates would be formed with the increase in nanoTiO_2 concentration in the medium. Aggregation can decrease the available surface area of nanoTiO_2 influencing their bioavailability and biological response of the tadpoles. Even in the natural habitats of the tadpoles, such aggregation of nanoTiO_2 can occur depending on the characteristics of the nanomaterial and the receiving waters.

In the 96 h exposure study, some nanoTiO_2 added to the aged tap water were settled on the bottom of the tanks due to the formation of TiO_2 aggregates. We observed that tadpoles fed upon these TiO_2 aggregates while grazing on the bottom of the tanks. Ingested aggregates were clearly visible externally as whitish solid particles in the curved gut of the tadpoles exposed to the ≥ 30 mg/L of nanoTiO_2 compared to the controls (Fig. 2a, b). Trapping of nanoparticles inside the digestive tract may prevent normal uptake of nutrients which can lead to physiological impairments at a later stage (Boura et al. 2015). At 96 h, survival of tadpoles exposed to nanoTiO_2 was not affected significantly ($p > 0.05$) (Table 1) implying lack of acute toxicity associated with the tested nanoTiO_2 concentrations. However post-exposure observations revealed the potential impacts of nanoTiO_2 pre-exposure. Upon transfer of these tadpoles to aged tap water, morphological deformities viz. abnormal swelling of the body (edema) and lateral deviation in the normally straight line of the spine (scoliosis) were observed (Fig. 2c, d) in some tadpoles pre-exposed to nanoTiO_2 during 10 and 14 days of post exposure. On the 10th day, edema was observed in 6%, 5%, 16% and 14% of the survived tadpoles pre-exposed to 10, 30, 50 and 100 mg/L nanoTiO_2 respectively whereas 5%, 8% and 14% of the survived tadpoles pre-exposed to 30, 50

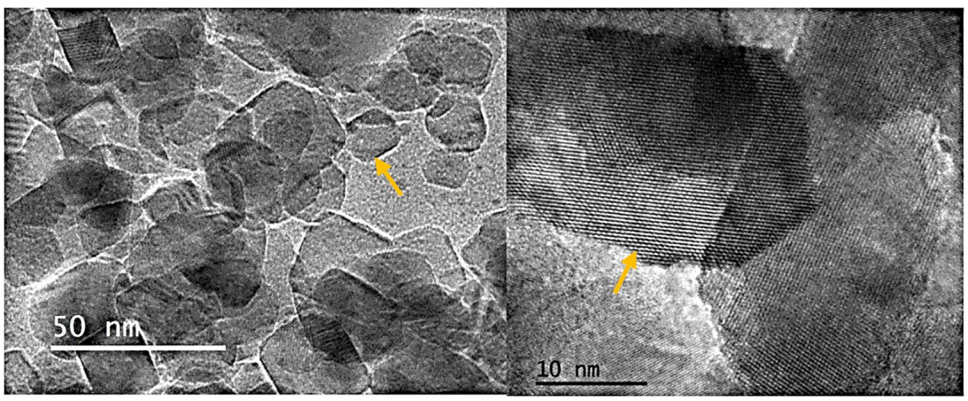

Fig. 1 Transmission electron microscopic images of the nanoTiO_2 used in this study. Arrow indicates a nanoparticle

and 100 mg/L nanoTiO$_2$ displayed the scoliosis condition. However, these tadpoles did not survive until 21 days. Survival of the tadpoles pre-exposed to nanoTiO$_2$ at the end of 21 days post-exposure was 7%–15% (Table 1) compared to the survival (63%) of the respective controls. Body lengths (SVL) of nanoTiO$_2$ exposed tadpoles were not significantly different from the controls (Table 2) probably due to the small number of survived tadpoles and greater variations of the body sizes of the individual tadpoles exposed to nanoTiO$_2$. However, based on the 'Gosner stage ranking' (McDiarmid and Altig 1999) of the survived individuals (Table 2), it can be deduced that there were delays in development of the tadpoles exposed to high concentrations (30–100 mg/L) of nanoTiO$_2$. Nations et al. (2011) reported that exposure of embryos of African clawed frog, *Xenopus laevis* to 0.001 mg/L to 1000 mg/L TiO$_2$ (32 nm) for 96 h

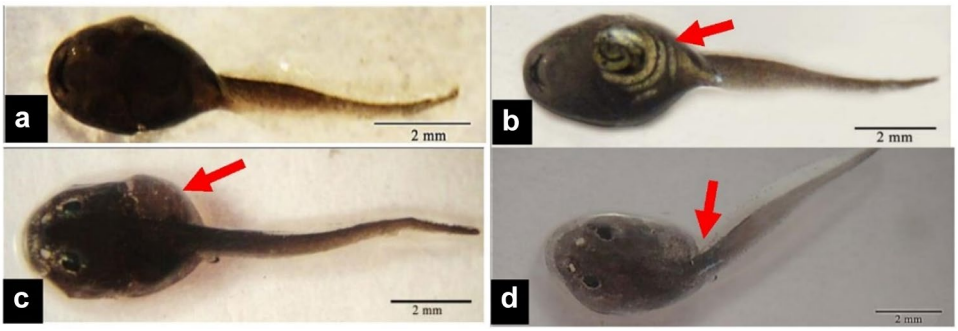

Fig. 2 Ventral view of *D. melanostictus* **a** control tadpole and **b** a tadpole exposed to nanoTiO$_2$ showing whitish nanoTiO$_2$ aggregates in the coiled gut (arrow). Morphological deformities (arrows) seen in *D. melanostictus* tadpoles transferred to nanoTiO$_2$ free water following 96 h exposure to nanoTiO$_2$: **c** abnormal swelling of the body (edema) and **d** lateral deviation in the normally straight line of the spine (scoliosis)

Table. 1 Survival of tadpoles of *D. melanostictus* during 96 h exposure to nanoTiO$_2$ and during 21 days post exposure to nanoTiO$_2$ free aged tap water

nanoTiO$_2$ concentration (mg/L)	Survival (%)				
	48 h exposure	96 h exposure	7 days post exposure	14 days post exposure	21 days post exposure
0 (control)	100 ± 0	93 ± 2	78 ± 2	67 ± 2	63 ± 2
5	98 ± 2	93 ± 2	57 ± 3*	18 ± 3*	7 ± 2*
10	98 ± 2	87 ± 2	55 ± 5*	25 ± 3*	15 ± 6*
30	98 ± 2	90 ± 5	57 ± 2*	28 ± 7*	13 ± 4*
50	95 ± 3	90 ± 6	51 ± 6*	23 ± 2*	12 ± 2*
100	98 ± 2	92 ± 3	52 ± 2*	28 ± 4*	8 ± 2*

Data are presented as mean survival ± SEM of three replicates with n = 20 tadpoles per replicate. In a column, the data with the asterisk indicates significant difference from the respective control ($p \leq 0.05$).

Table. 2 Snout to vent length (SVL) and development stage of tadpoles of *D. melanostictus* after 21 days post exposure to nanoTiO$_2$ free aged tap water

nanoTiO$_2$ concentration (mg/L)	SVL (cm)*	Total survivors	Tadpoles with hind legs	Tadpoles with fore legs	Developmental stage (Gosner stage)
0 (control)	1.80 ± 0.01 (1.8–2.1)	38	27	0	39–41
5	1.80 ± 0.15 (1.5–2.1)	4	2	1	40–42
10	1.68 ± 0.04 (1.4–2.2)	9	3	1	40–42
30	1.65 ± 0.03 (1.4–2.0)	8	1	0	38
50	1.77 ± 0.09 (1.6–2.0)	7	3	0	38
100	1.65 ± 0.05 (1.4–1.9)	5	0	0	< 36

The SVL data of nanoTiO$_2$ tadpoles are not significantly different from the control ($p > 0.05$)

*SVL data are presented as mean ± SEM and range

had no effects on mortalities or frequencies of malformations but inhibited growth (SVL) at the highest concentration (1000 mg/L). However follow up observation component had not been incorporated into their exposure design in order to evaluate post-exposure effects of the short term exposure.

In a chronic exposure study, Zhang et al. (2012) reported decreases in survival of *X. laevis* tadpoles at 14 days exposure to nanoTiO_2 (5, 10, 32 nm) alone or in combination with ultraviolet light. High-concentration TiO_2 also affected growth of *X. laevis* tadpoles and delayed developmental stages (Zhang et al. 2012). In the present study, the survival of *D. melanostictus* exposed to nanoTiO_2 was decreased with time during the 14 days chronic exposure period (Table 3). At the end of 14 days, mortalities of the tadpoles exposed to 0.1, 0.3, 1, 3 and 10 mg/L nanoTiO_2 were 43%–40% which were significantly different ($p < 0.05$) from the mortalities (17%) of control tadpoles. However chronic ecotoxicity threshold concentrations for lethality (e.g. 14 day LC50, LC10 values) could not be estimated due to inadequate partial effects for concentration-response relationships. Based on the nominal concentrations, no observed effect concentration for mortality at 7 days was 0.1 mg/L whereas lowest observed effect concentration at 7 days and 14 days were 0.3 mg/L and 0.1 mg/L respectively. Although survival of tadpoles exposed to 10 mg/L nanoTiO2 was not significantly different from the controls after 96 h exposure (Table 1), 14 days exposure (Table 3) caused a significant reduction in the survival (57%) compared to the controls (83%). Long-term exposure may have increased the bioavailability of nanoTiO_2 changing the biological responses of the tadpoles. Moreover, the survival rates of the tadpoles under short term and chronic exposures (Tables 1, 3) were not dependent on the exposed concentration of nanoTiO_2. This may be attributed to the formation of much larger aggregates of nanoparticles in the exposure media with the increase in nanoTiO_2 concentrations reducing their bioavailability. AChE is the key enzyme in cholinergic transmission in the nervous system and its inhibition may indicate neurotoxic effects (Russomt et al. 2014). In the present study, chronic exposure of nanoTiO_2 for 14 days caused an apparent decrease in the AChE activity of the *D. melanostictus* tadpoles (20%–36% inhibition) compared to that of the respective control (Table 3). However the differences were not significantly different

Histological examination of the intestinal tissues revealed damaged areas of the intestinal epithelium in the three examined tadpoles exposed to each concentrations of nanoTiO_2 compared to the controls (Fig. 3a, b, c). In addition dark colour aggregates were found inside the intestinal epithelial cells of these nanoTiO_2 exposed tadpoles. This may be attributed to the accumulation of ingested nanoTiO_2 aggregates within the cells which warrants further studies. Gitrowski et al. (2014) reported that nanoTiO_2 can be directly taken up by the epithelium of the gut through endocytosis related pathways depending on the crystal structure. Accumulation of nanoTiO_2 in different organs may lead to physical stress and tissue damage (Vale et al. 2016). Gut wall of the examined tadpoles exposed to ≥ 3 mg/L nanoTiO_2 was shrunken which may be due to the pressure of trapped TiO_2 aggregates within the gut lumen. The liver tissue of the three examined tadpoles (Fig. 3d, e, f) exposed to all concentrations of nanoTiO_2 exhibited prominent melanomacrophages. In addition, pycnotic nuclei with disintegrated hepatocytes were found in the livers of all the examined tadpoles exposed to ≥ 1 mg/L nanoTiO_2. These alterations may be due to the stress caused by chronic TiO_2 exposure. To the best of our knowledge, this is the first report on histological alterations of amphibian larvae induced by nanoTiO_2 exposure. The overall results based on combined short term and chronic exposure of *D. melanostictus* tadpoles show that release of nanoTiO_2 to the aquatic ecosystems could cause negative impacts to the amphibian populations.

Table 3 Survival of tadpoles of *D. melanostictus* during 14 days chronic exposure to nanoTiO_2 and acetylcholinesterase (AChE) activity at 14 days exposure

nanoTiO_2 Concentration (mg/L)	Survival (%)		AChE activity (nmol/min/mg protein)	Inhibition of AChE activity
	7 days	14 days		
0 (control)	97 ± 2	83 ± 3	134 ± 19	–
0.1	80 ± 5	60 ± 6*	107 ± 14	20%
0.3	75 ± 5*	60 ± 5*	101 ± 12	25%
1.0	73 ± 3*	57 ± 2*	100 ± 35	25%
3.0	75 ± 3*	60 ± 6*	101 ± 16	25%
10	72 ± 2*	57 ± 3*	86 ± 14	36%

Data are presented as mean ± SEM of three replicates (n = 20 tadpoles per replicate for survival data and 6–8 pooled tadpoles per replicate for AChE data). In a column, the data with asterisk indicate significant difference from respective control ($p \leq 0.05$)

Conclusions

Short-term (96-h) exposure to a range of concentrations (5–100 mg/L) of nanoTiO_2 (anatase form, particle size < 25 nm) had no significant lethal effect on *D. melanostictus* tadpoles. However, pre-exposed tadpoles showed significant mortalities compared to the controls upon transfer to nanoTiO_2 free aged tap water for another 21 days. Moreover the tadpoles exposed to 30–100 mg/L nanoTiO_2 displayed morphological deformities (scoliosis and edema) and developmental delays. Chronic exposure of tadpoles to 0.1, 0.3, 1, 3 and 10 mg/L nanoTiO_2 for 14 days induced significant mortalities compared to the control tadpoles. At the end of 14 days, significant inhibition of AChE activity

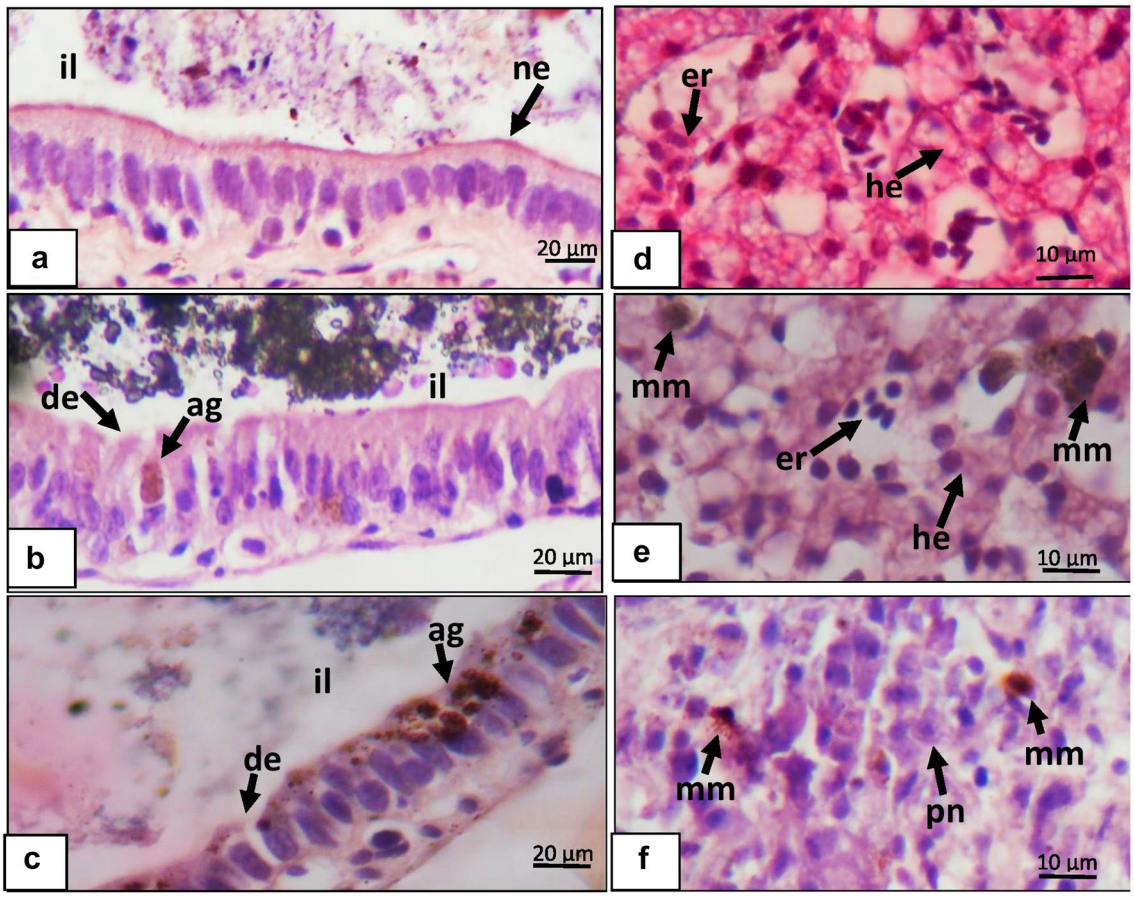

Fig. 3 Histological images of the intestine (**a**, **b**, **c**) and liver (**d**, **e**, **f**) tissues of *D. melanostictus* tadpoles. **a**, **d** control tadpole; **b**, **e** tadpoles exposed to 0.1 mg/L nanoTiO_2 for 14 days; **c**, **f** tadpoles exposed to 1 mg/L nanoTiO_2 for 14 days. il: lumen of intestine with ingested food/particles; ne: normal intestinal epithelium, de: damaged intestinal epithelium, ag: internalized dark aggregates within epithelial cells, he: hepatocytes, er: erythrocytes, mm: melanomacrophage, pn: hepatocytes with pycnotic nuclei

was not found in the nanoTiO_2 exposed tadpoles. Histological examinations of the tadpoles exposed to nanoTiO_2 for 14 days showed structural alterations in the intestine and liver tissues compared to the control tadpoles. The overall results show that release of nanoTiO_2 to the aquatic ecosystems may induce negative impacts to the amphibian populations. This study recommends the use of tadpoles of *D. melanostictus* as an amphibian model to investigate nanoecotoxicity under tropical conditions.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest. The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval All applicable international guidelines for the laboratory maintenance of animals and use of animals were followed.

Informed Consent Informed consent is not applicable in this study.

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