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Ecotoxicological effects of titanium dioxide nanoparticles on the freshwater mussel *Lamellidens marginalis*: physiological disruption, oxidative stress, and ecological implications

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Titanium dioxide nanoparticles (TiO_2 NPs) are widely distributed in aquatic environments due to their extensive industrial and commercial applications. Several studies have reported the adverse effects of TiO_2 NPs on aquatic organisms; however, limited information is available regarding their impact on the freshwater mussel *Lamellidens marginalis*. The present study investigates the physiological and biochemical responses of *L. marginalis* following acute exposure (7 days) to varying concentrations of TiO_2 NPs (Control, 5.0, 50, and 100 µg/L). Physiological parameters, including the condition index, filtration rate, and clearance rate, were assessed alongside hemocyte count, metabolic activity (electron transport system activity), and energy reserve content (glycogen, lipid, and protein levels). Additionally, oxidative stress biomarkers, including antioxidant enzyme activity, biotransformation enzyme activity, and lipid peroxidation levels, were evaluated. Results revealed a significant accumulation of TiO_2 NPs in the gill tissues, accompanied by a marked decline in filtration rate and total hemocyte count, along with an increase in nitric oxide production. Exposure to higher concentrations of TiO_2 NPs resulted in substantial alterations in energy reserve levels and oxidative stress biomarkers, indicative of metabolic disruption. Furthermore, mussels exposed to elevated TiO_2 NP concentrations exhibited reduced feeding activity and energy expenditure, leading to impaired physiological performance, including potential consequences for growth and reproduction. Histopathological analysis demonstrated pronounced gill damage in mussels from the higher exposure groups. These findings emphasize the ecological risks associated with TiO_2 NP contamination and underscore the need for stringent measures to mitigate their impact on freshwater bivalves.

Keywords *Lamellidens marginalis*, Condition index, Hemocyte, Cellular damage, Histology, TiO_2

The pioneering properties of nano titanium dioxide (nano-TiO_2) make their presence in the production and usage of consumer and personal protection products[1]. TiO_2 possesses some peculiar characteristics to attract industrialists, and it includes low cost, availability, antimicrobial activity, white color, longstanding stability, great hydrophilicity, block UV light and is preferred for the production of paints, plastics, creams, toothpaste, paper, and food packaging[2–9]. As an effective catalytic agent and surface-assimilative of biological pollutants and heavy metallic element it was used in eco-friendly nano remediation. This significant input of nano-TiO_2

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makes them accumulate in the aquatic environment via urban and industrial sewage in the concentration of μg/L (0.7–16 μg/L)[1]. The entry of TiO_2 occurs accidentally in the aquatic environment worldwide by their interaction between contaminants in consumer products that may perhaps amend the ecological behaviour and dispersal of human-caused contaminants, especially to aquatic organisms. Nevertheless, in aquatic systems, TiO_2 frequently occurs in very low concentrations in the range of 0.01 and 5.5 μg/L which increases dramatically in the upcoming years[10,11]. Normally the presence of TiO_2 (100–300 μg/L) is reported in household sewage, manufacturing run-offs, and effluents from the paints of buildings[12]. Besides, nano-TiO_2 will be taken by aquatic organisms along with other ecological toxicants such as metals, xenobiotics, and pollutants which mean habitually bioaccumulation and toxicity level increases in the aquatic organisms[13]. The adverse effect of nano-TiO_2 on aquatic pelagic and benthic invertebrates has been reported[8]. Immunotoxicity, oxidative anxiety, and cytotoxicity in addition to biological and generative amendments were studied in freshwater organisms[1]. The occurrence of nano-TiO_2 was reported in the mediterranean mussel *Mytilus galloprovincialis*[14]. In the freshwater fish, *Cyprinus carpio* nano-TiO_2 seems to be accumulated in the intestinal tract and on lamellae tissue[15,16]. Similarly, nano-TiO_2 cause sublethal antagonistic impacts on the premature hatching and anomalous growth in *Oryzias latipes*[17]. Accumulation and intake of nano-TiO_2 by marine mammal bottlenose dolphins results in the genotoxicity of leukocytes[18,19]. However, as far as we are concerned, in the previous literatures the harmfulness provoked by nano-TiO_2 in the freshwater mussels *L. marginalis* has not been investigated yet. Conversely, preceding experiments on the marine mussels *M. galloprovincialis* show significant changes in their metabolic capacity, oxidative damage, and defense mechanisms after exposure to titanium (Ti)[20]. Wang et al.[21] reported that nano-TiO_2 alters the hemocyte biomarkers including hemocyte count, mortality, phagocytosis activity, lysosomal content, esterase activity, mitochondrial number, and reactive oxygen species content. Nano-TiO_2 shows negative impacts on the gill of mussels *M. coruscus* by increasing their malondialdehyde (MDA) concentration[22]. Therefore, impacts of nanoTiO_2 in marine organisms and their interaction with the marine environment were reported well while their impacts on the freshwater organism are still scarce and the need for more studies is required. Among various aquatic organism's bivalves were considered a vital model organism for monitoring aquatic pollution[23–26]. The freshwater mussel *L. marginalis* was specifically selected as a study organism owing to their feeding by filtering out floating particles, obscuring the effects of pollutants like NPs on the water. Using bivalves as a model organism in nanotoxicology studies has been successful[24].

In these aspects, the current experiment intends to assess the short term (7 days) impact of nano-TiO_2 in the freshwater mussel *L. marginalis* to determine the physiological activity (condition index, filtration rate, clearance rate and hemocyte enumeration), metabolic capacity (electron transport system activity), energy reserves content (glycogen and protein concentrations), oxidative biomarkers (antioxidant and biotransformation enzymes activities, lipid peroxidation levels) and histological alterations in gills.

Materials and methods

Preparation and characterization of TiO_2

TiO_2 was prepared manually in the laboratory by a simple precipitation method using titanium isopropoxide ($C_{12}H_{28}O_4Ti$) as a starting material. Here, titanium isopropoxide (5 ml) was gradually added in drops to double distilled water (8 ml) which hydrolysis the alkoxide to form a precipitate of titanium oxides. Thus, the obtained solution was stirred continuously (40 °C for 30 min) and the white precipitate formed was collected after centrifugation by washing with deionized water and methanol followed by drying in a hot air oven (80 °C). The fine powder was subjected to characterization using UV–Visible spectrophotometer at the resolution of 1 nm from 200 to 800 nm, FTIR spectrum was viewed in FTIR spectroscopy at a resolution of 500–400 cm^{-1}, XRD- X-ray diffraction pattern and shape and size of chemically synthesized TiO_2 were determined by means of HR-TEM (High-Resolution Transmission Electron Microscopy). In addition, Dynamic light scattering (DLS) visually represents particle size distribution relative to intensity in terms of a percentage. A DLS analyzer was employed for size distribution (Analyzer Litesizer 500). Zeta-potential is the electrical potential at the solid–liquid interface caused by the relative mobility of the nanoparticle and the solvent. In order to determine the zeta potential, TiO2 were combined with distilled water.

Experimental design

Lamellidens marginalis were collected from a local freshwater pond in Karaikudi, Tamil Nadu (India), with a mean shell length of 3.7 ± 3.9 cm and shell width of 2.8 ± 3.0 cm. The mussels are carefully transported from the pond to the experimental laboratory and maintained for acclimation in the laboratory condition with continuous aeration for ten days. The biophysiochemical parameters like temperature 28.4 ± 1.2 °C, salinity 0.25 ± 0.05 ppm, pH 7.9 ± 0.2, and dissolved oxygen 6.8 ± 0.4 mg/L with a photoperiod of 12 h light:12 h dark were maintained constant[27]. Freshwater plankton (20 mg/L Spirulina) were given to the mussels as feed for every 2–3 days[28]. After 10 days of acclimatization subsequently, mussels were separated in experimental tanks of 20 L capacity with forty animals in each group (twenty individuals per tank), considering 3 treatment groups and one control: A-Control (0), B-5.0 μg/L TiO_2, C-50 μg/L TiO_2, D-100 μg/L TiO_2. The concentrations of TiO_2 in this experiment selected were environmentally relevant as per earlier reports to cause lethal impacts on aquatic organisms[29]. During the experimental duration (7 days) all the physicochemical parameters of the water were regularly (2 days once) checked and maintained. Throughout the study duration, the water was renewed 2 days once, all requirements were reset in each tank. In the treated tanks water samples (5 ml) were obtained from each tank for TiO_2 quantification every 2 days after spiking. During the termination of the 7th day of experimentation, mussels were sacrificed for studying TiO_2 accumulation, metabolic capacity, energy reserves, physiological, biochemical, and histological analysis. There was no fatality at the end of the experimentation. 2 animals from each tank (4 per group) were used for TiO_2 accumulation, 8 animals (16 per group) for physiological parameters, 2 animals from each tank (4 per group) for metabolic capacity and energy reserves, 3 animals from each tank (6 per group) for biochemical activities and 1 animal (2 per group) for histological analysis were used. To study the above parameters, gill was selected as the target organ for this study as gill are the primary target organ for detecting pollutants in water. Moreover, as filter feeders, mussel's uptake food by filtering the water and the sensitive gill tissues easily detect pollutants in the water column[30].

Ti quantification in water and gill tissue

For Ti quantification in water, samples of 5 ml/tank (2 samples per group) from each treated tank were collected immediately after spiking to access real exposure concentration, and before each water renewal. Water samples were also collected from blanks after spiking and before each week renewal. Regarding tissue, 1 animal from each tank (2 per group) was collected. The gill tissues were dissected out from mussels and desiccated in a hot air oven to determine the quantity of Ti accumulated. The tissue was processed as per the detailed procedure of Gobi et al.[27] in which a digestion falcon tube containing 3 mL of nitric acid was taken to which 0.2 g of dried tissue sample was added (2 animals from each tank (4 per group)). Following a 1-h digestion process in a water bath, in an optimum room temperature it was cooled, via filter paper it is further filtered, and the solution obtained was diluted up to 10 ml using distilled water. The same procedure was repeated for water samples also. Using Atomic Absorption Spectrophotometer (AAS) the amount of Ti accumulated in gills and the concentration of Ti in the treated water were evaluated.

Physiological parameters

Condition index

The Condition Index (CI) activity was measured corresponding to the enhanced method of Matozzo et al.[31]. CI is recognized as a standard technique to determine the general physiological status of animals. The tissues of mussels (2 animals per tank i.e., 4 per group) were cautiously detached from shells and rinsed in distilled water. Further, the tissue and shells were dried for 48 h at 60 °C in a hot air oven and weighed. This was calculated in percentage as per the below formula.

$$\mathrm{CI}\,(\%) = \frac{\text{dry weight of the soft tissues (g)}}{\text{the dry weight of the shell (g)}} \times 100$$

Estimation of filtration rate

The filtration rate of gills was determined both in control and TiO_2-treated animals. Two animals per tank and 4 per group were used to determine the filtration rate subsequent to the procedure of Ray et al.[32]. The animals were kept in a tank with 800 mL of a hydrous medium of neutral dye (1 mg/L of water). The filtration rate was calculated by the beginning and concluding concentrations of neutral red dye in water after reading the OD at 550 nm using a spectrophotometer. This was done based on the beneath formula.

$$\text{Filtration rate } = \frac{M}{\mathrm{n.t}} \log \frac{\mathrm{C_0}}{\mathrm{C_t}}$$

here, M = volume of water; n = number of mussels; t = time in hour; C_0 = initial dye concentrations; Ct = final dye concentrations.

Clearance rate

Enumerating the clearance rate (CR) both in control and treated animals were done according to the enhanced procedure of Almeida et al.[33]. CR was determined in accordance with the rate of neutral red dye absorbed by the animals. CR helps to estimate by what meansTiO_2 affects the mussel's clearance rate compared to the control condition. The animals (2 animals per tank and 4 per group) were kept in a tank with an aqueous solution of Congo red solution (15 mg/L) and the CR was calculated by the initial and final concentrations of Congo red solution in water after reading the OD at 498 nm using a spectrophotometer. The clearance rate was calculated using the below formula.

$$\text{Clearance rate } = \frac{\mathrm{V}}{\mathrm{nt}} \times \log\left(\frac{\mathrm{C_i}}{\mathrm{Ct}}\right)$$

were,

V = volume in the container.
n = number of mussels.
t = time in hour.
C_i = initial dye concentrations.
Ct = final dye concentrations.

Collection and enumeration of hemocytes

During the termination of the 7th-day of experimentation to 5.0, 50, and 100 µg/L TiO_2 and non-treated control 2 animals per tank i.e., 4 per group were collected to determine hemocyte quantity. Approximately about 2–3 ml of hemolymph was withdrawn from the adductor muscles to examine the hemocytes following the technique of Ray et al.[32]. Thus, collected hemocytes will be centrifuged at 4000 rpm for 10 min at 4 °C. After centrifugation, the obtained hemocyte pellet was resuspended in sterile saline solution. In addition, the viability (live or dead) of the hemocytes was detected using staining with 0.4% trypan blue and counted through a Neubauer hemocytometer to analyze the number of alive cells and entirety hemocytes count was measured in 10^6 cells/ml.

Sample preparation

Mussel samples collected after experimentation were processed for further physiological and biochemical studies. Instantly, after dissecting tissue (gill) samples (0.5 g/individual) were immediately homogenized in PBS (phosphate buffer saline) and extracted with specialized buffers (1:2) for every biochemical marker[33,34] by means of a mortar and pestle. The extracted samples were centrifugated for 20 min at 5000 rpm and the supernatant was stocked at – 20 °C.

Metabolic capacity and energy reserves

Gills of 2 animals from each tank (4 per group) for determining metabolic capacity and energy reserves were grinded using a mortar and pestle and divided into aliquots of 0.5 g fresh weight (FW). Specific buffers (1:2) were used for different biomarkers. Based on the method of Coen and Janssen[35] electron transport system (ETS) activity was determined. Depletion of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to iodonitrotetrazolium formazan (INT-formazan) was read at 490 nm. The activity of ETS was determined by the quantity of formazan developed using an absorption coefficient (Ɛ) of 15,900/M/cm, and the outcomes were articulated as nmol/min/g.

Succeeding the phenol–sulphuric acid method of Yoshikawa[36], the amount of glycogen (GLY) was analyzed. Glucose was used (0–5 mg/ mL) to acquire a standard curve. The optical density was deliberated at 492 nm and expressed in mg/g.

Following the technique of Robinson and Hogden[37], using bovine serum albumin (BSA) as standard (0–2 mg/ mL) total protein (PROT) content was quantified. The PROT content was estimated at 540 nm and the outcomes were determined in mg/g.

Total lipids (LIP) were revealed succeeding the method of Cheng et al.[38] where cholesterol is used as standard (0–100%). The LIP content was analyzed in 540 nm and the data were articulated in percentages.

Biochemical antioxidant enzymes

Biochemical markers were measured using 3 animals from each tank and 6 per group. The gill tissue was processed corresponding to the procedure of Almeida et al.[33,34]. SOD biomarker was measured based on the procedure described by Suzuki[39]. The amount of xanthine oxidase (XO) reduced into nitro blue tetrazolium (NBT) was visualized at 560 nm. The result was expressed as µ/mg. The activity of total CAT was done following a decomposition method by Cohen et al.[35]. The amount of hydrogen peroxide (H_2O_2) decomposed was determined at 405 nm and the results were stated in µ/mg. GST activity was quantified according to the adaptation method of Habig et al.[40]. Biomarker of GST were performed by the development of thioether due to the coupling of substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione. GST was read spectrophotometrically at 340 nm and the results were expressed as µ/mg. GPx was determined following Rotruck et al.[41] in which NADPH corrosion occurs in the existence of H_2O_2 that is measured at 420 nm and stated in terms of U per mg protein.

Indicators of cellular damage

Cellular damage, LPO, was evaluated succeeding the procedure of Buege and Aust[42]. By-product malondialdehyde (MDA) formed was quantified and expressed in µmol of MDA formed as µmol MDA/mg. The activity of PCA was examined by pursuing the technique of Reznick and Packer[43]. The development of dinitrophenylhydrozones was examined at 360 nm owing to the presence of p2,4-dinitrophenylhydrazine and the results are expressed in µmol carbonyl/mg.

Determination of nitric oxide generation

At the end of 7th day of experimentation 2 animals per tank i.e., 4 per group were collected to determine the amount of nitric oxide generation. Almost about 2–3 ml of hemolymph was collected from the adductor muscles to examine nitric oxide generation following the method of Ray et al.[32]. The production of nitric oxide was analyzed based on the method of Green et al.[44], using Griess reagent (0.1% naphthyl ethylenediamine dihydrochloride and 1% sulphanilamide in 5% concentrated orthophosphoric acid) reaction. The amount of nitrite released was read spectrophotometrically at 550 nm and the findings are stated as µM nitrite/10^6 cells/ min.

Histological analysis

Histological studies were performed in the gill of 1 mussel per tank 2 per group. At the end of the experimental duration mussels' gills were dissected and damages to gill lamellae structure, ciliated epithelium, interlamellar junction, and water tubes were analyzed following the procedure of Stalin et al.[45] with slight modifications by Khan et al.[46] and Khudhur et al.[47]. Gill samples was fixed 24 h using formalin preservative and washed 2 h with flowing pipe water. They are desiccated and implanted in paraffin blocks and using haematoxylin and eosin. Finally, with a bright field light microscope, the samples were inspected.

Statistical analysis

The acquired data (mean ± standard deviation) were evaluated to examine the significant variations between treatments for each biomarker using Microsoft Excel and one-way ANOVA (SPSS, 16.0 Package). The level of significance was $p \leq 0.05$, and significant differences were recognized in the figures with different alphabetic letters.

Result and discussion

TiO_2 characterization

The synthesized nanoparticles in the present study reported an enhancement of UV absorption with a high-pitched peak extinction coefficient at 350 nm (Fig. 1a). The FTIR spectroscopy proved the capping and attributed compounds in synthesised TiO_2 (Fig. 1b). X-ray diffraction confirmed the existence of monophasic particles with rutile crystallography and the purity of the corresponding phase. Increasing calcination temperature increases the crystallinity of TiO_2 and converts it to the rutile phase. Figure 1c shows the position of the rutile reflections phase composition of titanium nanoparticles by the XRD analysis (JCPDS No. 01-077-0441). Particle geomorphology and size of TiO_2 were observed by HR-TEM microscopy with spherical structure and sizes ranging from 5 to 15 nm (Fig. 1d). Fine particles and molecules are in continual random thermal motion, known as Brownian motion, which states that smaller particles diffuse faster than bigger ones. Implementing a particle

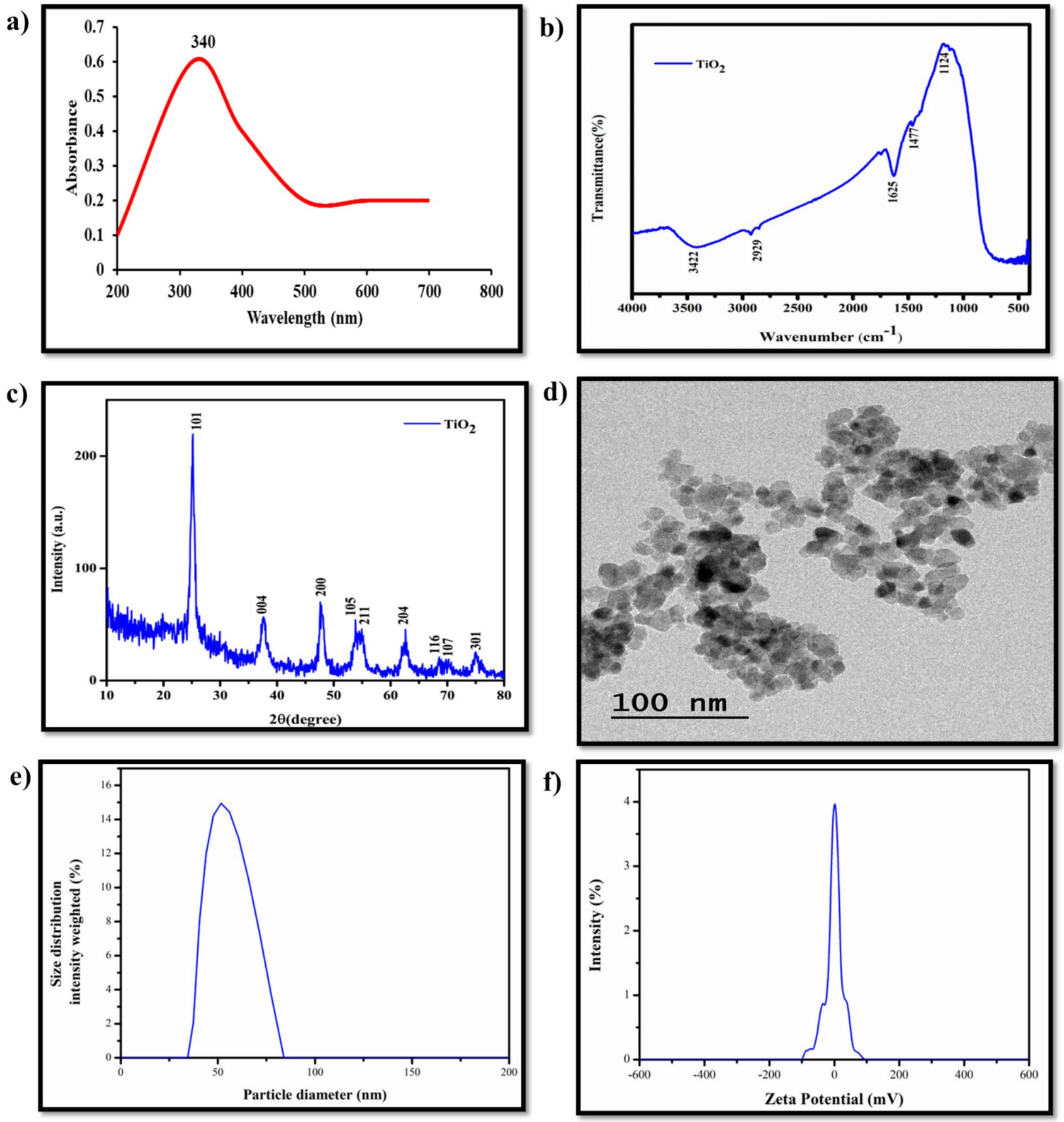

Fig. 1. Characterization of TiO_2: (**a**) UV–Vis spectrophotometer (UV), (**b**) Fourier Transform Infrared Spectroscopy (FTIR), (**c**) X-ray diffraction (XRD), (**d**) high resolution transmission electron microscope (HR-TEM).

size analyzer to determine the average diameter of TiO_2. As indicated in Fig. 1e, the average particle diameter measured by DLS method is 6 nm. Synthesized TiO_2 zeta potential dispersion is shown in Fig. 1f. The zeta potential value of the chemically produced TiO_2 is discovered to be − 29.6 mV, and the conductivity is measured to be 0.883 mS cm^{-1}. TiO_2 are less likely to clump together if they have significant electric charges attached to their surfaces. All of the tested concentrations of TiO_2 seemed stable over the 7 days of the experiment, and the nanoparticles did not stick together since the TiO_2 were stirred and sonicated before they were used. We tested the stability of TiO_2 over a period of 7 days using DLS and zeta potential. This shows that the TiO_2 are stable overall. The presence of strong electric charges on the particles' surface suppresses the agglomeration of AgNPs by keeping them away from each other. Consequently, the findings obtained are consistent with those previous reports[29,48].

Ti quantification in water and gill tissue

Accumulation of Ti in water from the exposure tanks is presented in Table 1 and the data obtained confirmed that the existent concentrations assessed in aquatic media stood close to the supposed concentration (2 μg/L). Higher values of Ti in the gills of *L. marginalis* were observed along the increased acquaintance gradient that was dependent on the exposure concentrations. A significant difference was obtained among the increased treated concentration (100 μg/L) and the other groups (Table 1) compared to the control. Gill tissue concentrations of Ti varied strongly between different exposure groups. The research carried out by Canli et al.[48] discovered that, the accumulation of Ti in the gill, digestive gland, and muscle of freshwater mussels *Unio tigridis* increased notably ($p < 0.05$) after exposure to 0, 1, 3, and 9 mg TiO_2 /L for 14 days. Similar to the above results, Shi et al.[49] experimentally proved that after exposure to 100 μg/LTiO_2 for 21 days the gills, foot, and mantles of blood clam (*Tegillarca granosa*), hard clam (*Meretrix meretrix*), and venus clam (*Cyclina sinensis*) accumulates higher level of Ti concentration. The findings of the present study and previous studies suggest that a higher concentration rate increases the accumulation of Ti in the bivalve species. The present study showed that polluting the aquatic environment with TiO_2 nanoparticle waste materials will lead to an increased accumulation of TiO_2 in freshwater mussels, which may be due to a synergic effect on the uptake of TiO_2 from the environment and the elimination of TiO_2 out of the body of mussels. Moreover, an increase in TiO_2 accumulation in mussels possibly will also be attributed to an increase in TiO_2 intake using other pathways other than filter-feeding[50].

Physiological parameters

Condition index

The influence of TiO_2 on the weight of mussels was enumerated by the condition index (CI).

The aggregation of TiO_2 in mussels' tissues was concurred, although not signed by a decrease in CI are presented in Fig. 2a. The results obtained reveal that 100 μg/LTiO_2 shows significantly lower CI than control, 5.0, 50 μg/L TiO_2. Furthermore, reduced CI levels with raising exposure concentrations, remarkably at the higher TiO_2 experimental group. The results obtained demonstrated that when bivalves are exposed to a polluted environment their body rhythm might force to decline, that could be indicated as a decrease in CI quantity. These outcome correlates with those acquired by Almeida et al.[33] subsequently experimenting marine clams *V. decussata* and *V. philippinarum* to 0.03, 0.30, 3.00, and 9.00 μg/L CBZ showing significantly lower CI in *V. philippinarum* than *V. decussata*. Gomes et al.[51] obtained no significant changes in CI after exposure to 10 μg/L of CuO NPs and Ag NPs for 15 days.

Filtration rate

A decrease in CR and CI was related to filtration rate (FR) as mussels are filter feeders. When exposed to polluted water dumped with contaminants including nanoparticles, mussels tend to keep shells close and avoid filtration which automatically decreases the body metabolism and FR value. The results obtained in the study make truth the premise as FR decreases significantly in all treated conditions of 5.0, 50, and 100 μg/L TiO_2 compared to the non-treated control. At greater concentrations (100 μg/L TiO_2) the rate of filtration decreased strongly which is shown in Fig. 2b. In supporting the premise and the obtained result of the present study, Ray et al.[32] observed inhibition in FR after exposing freshwater mussel *L. marginalis* to 5 mg of CuO NP and copper sulphate/L for 7 and 14 days as 27.267 ± 2.878 mL/indiv/h and 38.256 ± 1.824 mL/indiv/h. Moreover, Basti et al.[52] reported, that TiO_2 and AuTiO_2 nanoparticles changed the physiology of clams by lowering filtration and respiration rate. A decrease in respiratory rate possibly will be an effect of declined energy expenditure.

Conditions	Water	Gill tissue
CNT	ND	ND
5 μg/L	2.1 ± 0.01^a	1.7 ± 0.00^a
50 μg/L	3.7 ± 0.00^a	2.5 ± 0.01^c
100 μg/L	4.5 ± 0.01^a	3.2 ± 0.00^a

Table 1. Concentrations of Ti (mg/g) in mussels' gill tissues and water samples after 7 days of exposure to each condition (CNT, 5, 50 and 100 μg/L) and both temperatures. Values are mean ± standard deviation. Significant differences ($p \leq 0.05$) among concentrations for each are represented with different letters (lower case letters). Data are expressed as mean ± SD. *ND* not detected.

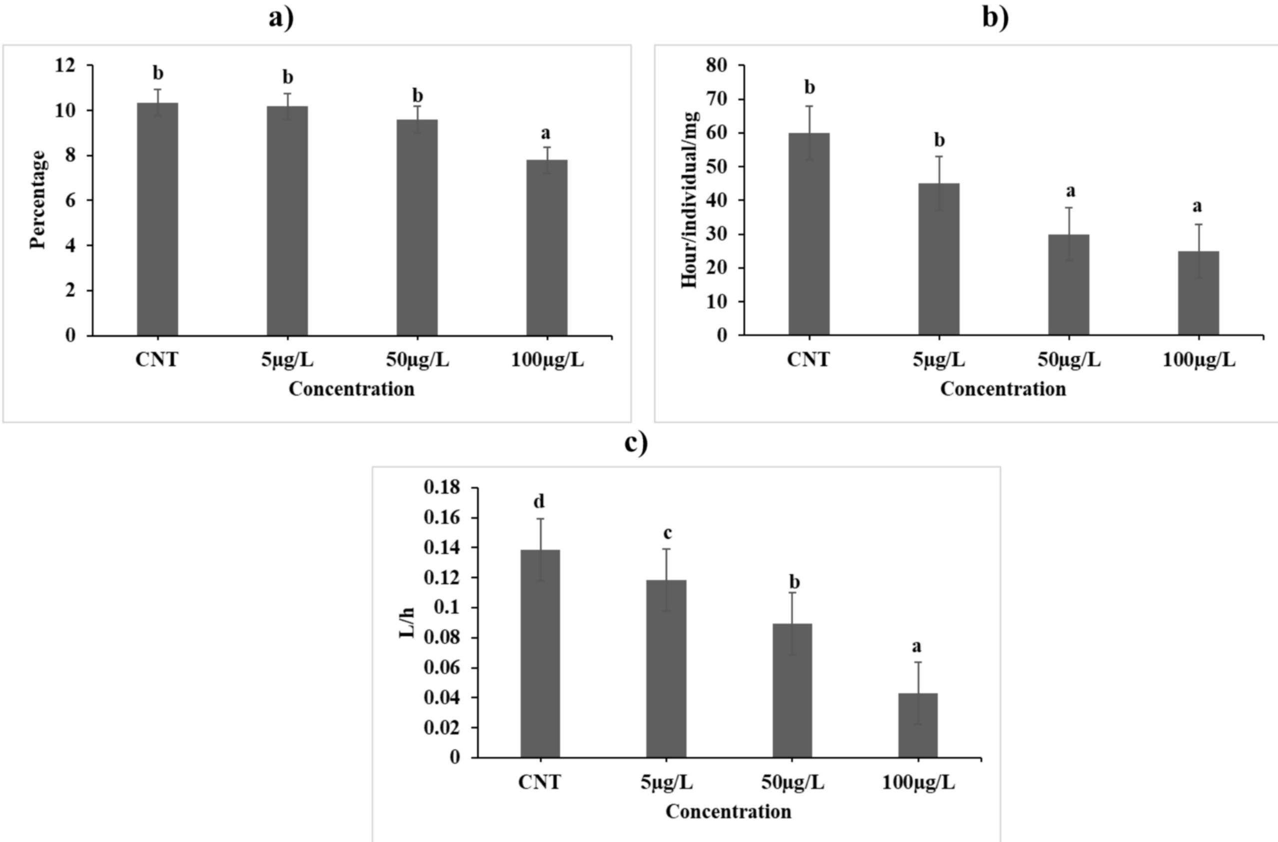

Fig. 2. Physiological parameters (CI, condition index; FR, filtration rate; CR, clearance rate) in *L. marginalis* exposed to TiO_2 after 7 days at each condition (CNT, 5, 50 and 100 µg/L). The data are represented as mean ± SD and significant differences ($p \leq 0.05$) among concentrations for each are represented with different letters (lower case letters).

Clearance rate

Clearance rate (CR) determines the purification endeavour of the mussels. Musses tend to shut their shells as an approach of safeguard once revealed to a traumatic environment. After exposed to pollutants mussels limits the filtration activity which decreases the CR rate. By doing so the metabolism of mussels decreases which automatically lowers the CI rate as mentioned above. The outcome of the current findings proves the above said premise as, upon exposure to higher concentration-100 µg/L of TiO_2 CR decreased significantly and shows lower CR than control, and other exposed groups with 5.0, 50 µg/L TiO_2 which is shown in Fig. 2c. The results obtained are related to those obtained by Almeida et al.[33] after exposing marine clams *V. decussata* and *V. philippinarum* to 0.03, 0.30, 3.00, and 9.00 µg/L CBZ shows significantly lower CR values. Similarly, a drop-in CR rate was obtained by Matozzo et al.[53], after 48 h exposure to the organic contaminant (4-nonylphenol) in *T. philippinarum*. Even though CR is the constituent of the energy budget it is extremely vulnerable to anxiety-tempted by pollutants. In the current findings, CR was drastically reduced at all TiO_2 exposed treatment groups compared to control by the mechanism of inhibiting oxidative metabolism due to a decrease in feeding.

Enumeration of hemocytes

Kacsoh and Schlenke[54] reported that, hemocyte density is widely employed as a marker to estimate the toxic stress, animal condition, and innate immune significance of invertebrates. In the present study, experimental exposure of TiO_2 for 7 days showed a noteworthy decrease in whole hemocyte count as $1.86 \pm 0.23 \times 10^6$ cells/mL respectively at the uppermost concentration of 100 µg/L followed by remaining exposure groups (5.0, 50 µg/L TiO_2) comparing to the control $4.92 \pm 1.12 \times 10^6$ cells/mL respectively (Fig. 3). As hemocyte acts as a biomarker for surveillance of the immune toxicological cells, a decrease in the number of hemocytes is threatening of increasing the intrinsic resistance of invertebrate[55]. Thus, the outcome of the existing experiment indicates an immune suppression in *L. marginalis* owing to a decrease in hemocyte count by altering immune competence after exposure to TiO_2 which disturbs the immune monitoring potential in bivalves. Exposure of 100 µg/L TiO_2 for 7 days resulted in an extreme decrease in whole hemocyte count as $1.86 \pm 0.23 \times 10^6$ cells/mL which is in understanding including the outcome found by Gagné et al.[56] who reported that after exposing freshwater mussel *Elliptio complanate* to Cd-Te quantum dots total hemocyte count was decreased that correspondingly suppressed the immune mechanism of the mussel. Moreover, an increase in the level of cell death, superoxide anions, and

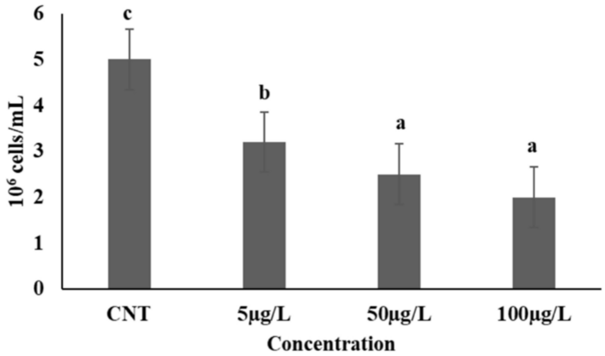

Fig. 3. Total count of hemocytes of *L. marginalis* exposed to TiO_2 after 7 days at each condition (CNT, 5, 50 and 100 µg/L). The data are represented as mean ± SD and significant differences ($p \leq 0.05$) among concentrations for each are represented with different letters (lower case letters).

Biomarkers	CNT	5 µg/L	50 µg/L	100 µg/L
ETS	0.3205 ± 0.06[a]	0.26025 ± 0.03[a, b]	0.13225 ± 0.05[b]	0.2350625 ± 0.08[c]
LIP	0.079 ± 0.04[a]	0.07405 ± 0.04[a]	0.0975 ± 0.01[a]	0.12425 ± 0.04[a]
PROT	0.04925 ± 0.00[b]	0.0525 ± 0.00[b]	0.0625 ± 0.01[b]	0.155 ± 0.02[a]
GLY	2.6875 ± 0.5[b]	3.0425 ± 0.4[a, b]	3.24 ± 0.2[a, b]	3.543 ± 0.06[a]

Table 2. Metabolic capacity and energy reserves; electron transport system (ETS), lipid content (LIP), protein content (PROT) and total glycogen level (GLY) in *L. marginalis* exposed to TiO_2 after 7 days at each condition (CNT, 5, 50 and 100 µg/L). The data are represented as mean ± SD and significant differences ($p \leq 0.05$) among concentrations for each are represented with different letters (lower case letters).

nitric oxides was observed by Tsiaka et al.[57] in the hemocytes of marine mussel *Mytilus galloprovincialis* after exposed to CBZ which affects the immune defense mechanism.

Metabolic capacity and energy reserves

After 7 days of experimentation, noteworthy lesser ETS activity was noted in mussels at the 50 µg/L TiO_2 treated group compared to control. Similarly, other treated groups (5, 100 µg/L TiO_2) also exert decreased ETS activity in contrast with the control group. Outcomes of the existing findings i.e., decreased ETS biomarker at the increased TiO_2 treated group specify that bivalves decrease their energy metabolism to avoid the accumulation of TiO_2 (Table 2). In support of our result, Monteiro et al.[58] obtained lower ETS activity after 7 days of exposure to marine bivalve *M. galloprovincialis* to 100 µg/L Ti while the activity increased after 14 days as they increased their metabolic capacity and defense mechanism to prevent Ti accumulation. In addition, Freitas et al.[59] prove that ETS activity suggestively declined in bivalves experimented with Multi-Walled Carbon Nanotubes (MWCNTs) (NP) and the amalgamation of both (As + NP) while increased in those treated with Arsenic (As) alone. This proves that cellular respiratory metabolism reduced after NP acquaintances (NP and As + NP), as bivalves show lesser ETS biomarker under those above-mentioned conditions compared to the exposure of As. Hence, the findings of the current study correlate with the fact that, by decreasing metabolic capacity (lower filtration rates associated with valve closure), mussels exposed to TiO_2 limited the accumulation of TiO_2. Decreased ETS activity increase the LIP, PROT, and GLY content in all treated groups (5, 50 µg/LTiO_2) compared to control while the highest level of energy reserves was obtained in the 100 µg/L TiO_2 concentration group (Table 2). Similar to the obtained results, Freitas et al.[59] obtained increased PROT and GLY content after 7 days of exposure of marine bivalve *M. galloprovincialis* to 100 µg/L Ti compared to the control. Similarly, Freitas et al.[59] obtained significantly increased PROT and GLY content in bivalves treated with Multi-Walled Carbon Nanotubes (MWCNTs) (NP) and in the combined exposed group (As + NP). Therefore, it's clear from the results that, a decrease in ETS activity was associated with an increased level of energy reserves, like LIP, PROT, and GLY content determining variations in the mussel metabolism.

Biochemical antioxidant enzymes

SOD, CAT, GPx, and GST activity in *L. marginalis* elevated with the increase of TiO_2 concentration, however extensively extreme levels were only observed for the concentrations of 50 µg/L TiO_2 after 7 days of experiment comparing the untreated group (Table 3). Mussels treated with 100 µg/L TiO_2 also shows increased SOD activity compared to the control while this level was lesser than those obtained for 50 µg/L TiO_2. Upon exposure to stressful conditions, mussels stimulate their oxidation inhibitor defenses, SOD, CAT, GPx, and GST enzymes, to inhibit the occurrence of cellular impairment (LPO, PCA levels), with the initiation of oxidative anxiety. Comparable to our result, in the marine clam *M. galloprovincialis* increased SOD, CAT, GPx, and GST activity

Biomarkers	CNT	5 μg/L	50 μg/L	100 μg/L
SOD	0.0773 ± 0.00[b]	0.0607 ± 0.00[b]	0.1783 ± 0.02[a]	0.1177 ± 0.07[a]
CAT	0.117 ± 0.00[a]	0.267 ± 0.00[a]	0.1297 ± 0.00[a]	0.1353 ± 0.01[a]
GPx	0.0763 ± 0.00[a]	0.07 ± 0.00[c]	0.0746 ± 0.01[ab]	0.064 ± 0.00[b]
GST	0.383 ± 0.04[a]	0.1526 ± 0.00[a]	0.201 ± 0.60[a]	0.2613 ± 0.00[a]

Table 3. Biochemical antioxidant parameters; superoxide dismutase (SOD), catalyse (CAT), Glutathione-S-transferase (GSTs) in gills of *L. marginalis* exposed to TiO_2 after 7 days at each condition (CNT, 5, 50 and 100 μg/L). The data are represented as mean ± SD and significant differences ($p \leq 0.05$) among concentrations for each are represented with different letters (lower case letters).

Biomarkers	CNT	5 μg/L	50 μg/L	100 μg/L
LPO	0.4233 ± 0.5[a]	0.542 ± 0.5[a]	0.4997 ± 0.05[a]	0.7607 ± 0.07[a]
PCA	0.234 ± 0.01[a]	0.197 ± 0.00[a]	0.235 ± 0.03[a]	0.252 ± 0.03[a]

Table 4. Cellular damage indicators; lipid peroxidation (LPO), protein carbonyl activity (PCA), in gills of *L. marginalis* exposed to TiO_2 after 7 days at each condition (CNT, 5, 50 and 100 μg/L). The data are represented as mean ± SD and significant differences ($p \leq 0.05$) among concentrations for each are represented with different letters (lower case letters).

were obtained at the increased exposure of 100 μg/L Ti after 7 days of exposure while the activity shows no significant difference after 14 days[58]. Results of Freitas et al.[59] confirmed the above facts by correlating with the results of the present study after exposing marine clams *Ruditapes philippinarum* to Multi-Walled Carbon Nanotubes (MWCNTs) (NP), Arsenic (As), and both (As + NP) for 28 days exposure period, the activity of SOD, CAT, GPx and GST was knowingly upsurged in polluted bivalves comparing to untreated control condition (CNT), with not at all any noteworthy alterations between bivalves treated with As, NP and As + NP. In addition to the above results, exposing marine clams *M. galloprovincialis* for 28 days to TiO_2, the SOD activity substantially raised beside the acquaintance grade, with the uppermost assessments in mussels at the increased treated condition of 100 μg/L TiO_2[29]. These results prove that the occurrence of TiO_2 generates overproduction of reactive oxygen species (ROS) and simultaneously, it limits the ability of clams to eradicate the surplus ROS via antioxidant mechanisms. Results of the present study indicate that the antioxidant enzymes (SOD, CAT, GPx, and GST) determined in this study functioned together in cells to remove excess ROS produced and struggle in contradiction of their toxic effects but remained insufficient to avoid cellular damage. Perusing the influences of $nTiO_2$, Canesi et al.[24] presented that *M. galloprovincialis* treated for 24 h significantly increased the CAT activity. Comparable outcomes were obtained by Barmo et al.[23] in the same animal after 96 h of experimentation.

Indicators of cellular damage

Overproduction of ROS, ensuing from TiO_2 exposure, was not absolutely eliminated and cellular damage occur. The results obtained show an increased LPO and PCA level after exposing the mussel to 100 μg/L TiO_2 for 7 days (Table 4). In all exposure conditions, extensively higher LPO and PCA levels were attained in mussels at 5 and 100 μg/L followed by 50 μg/L TiO_2 in association to the control circumstances. At every TiO_2 concentration, certainly not any considerable variations were detected in control among experimental phases. By our result, Monteiro et al.[58] obtained higher LPO levels after the 7th and 14th days of exposure of marine bivalve *M. galloprovincialis* to 50 and 100 μg/L Ti with no significant differences between both exposure periods. In addition, results of Freitas et al.[59] show significantly higher LPO and PCA levels in bivalves treated to Multi-Walled Carbon Nanotubes (MWCNTs) (NP) comparing to control organisms (CNT), with noteworthy alterations between bivalves treated with As, NP, and As + NP conditions. The obtained results were related to the inadequacy of defense mechanisms in bivalves during stress circumstances that were not able to trigger competently their defence mechanisms. Previous findings confirmed the ability of pollutants to induce cellular damage in bivalves by increasing LPO concentrations than PCA in the bivalve *R. philippianrum*, *C. grayanus*[58,59]. Deviations in the LPO cause alterations in creatures subjected to $nTiO_2$[60] in the fish *Oncorhynchus mykiss* for 14 days, while Xiong et al.[61] perceived enhanced LPO levels in *Danio rerio* animals treated with $nTiO_2$ after 96 h experimentation. The current facts demonstrated that accompanying antioxidant levels the cellular damages LPO and PCA cause adverse effects.

Nitric oxide generation

Considerable inhibition in the nitric oxide generation was obtained in *L. marginalis* after exposing mussel to 100 μg/L TiO_2 for 7 days. No significant difference was obtained between the remaining conditions associated to the control group. This proves the fact that gill muscular contraction was influenced by pollutants which decrease the rate of lamellary filtration and hence accumulation of pollutants occurs that alters ciliary beating and muscular changes further regulated by the nervous system[62]. Exposure to TiO_2 causes a decrease in the filtration rate of *L. marginalis* resulting in a reduction in oxygen intake and filter-feeding potential of the mussel scattered in a polluted environment (Fig. 4). The outcome of this study specifies the decline in lamella performance and

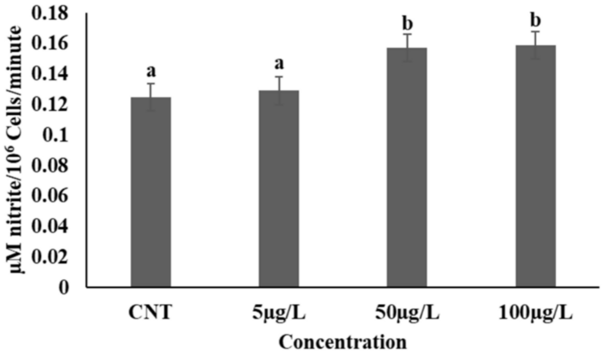

Fig. 4. Generation of nitric oxide in the hemocytes of *L. marginalis* exposed to TiO_2 after 7 days at each condition (CNT, 5, 50 and 100 μg/L). The data are represented as mean ± SD and significant differences ($p \leq 0.05$) among concentrations for each are represented with different letters (lower case letters).

metabolic anxiety in the investigational bivalves due to the exposure of animals to nanotoxicity. Similar to our results, Ray et al.[32] obtained suppression in the generation of nitric oxide in *L. marginalis* after CuO NP treatment which was interrelated with the reduction rate of gill filtration in *L. marginalis*.

Histological analysis

The outcome acquired confirmed that TiO_2 provoked variations in the lamellae of contaminated mussels. Histological damage of the gills of freshwater mussel *L. marginalis* after being treated with TiO_2 for 7 days is expressed in Fig. 5. The illustrations specify the location of rigorous lamella destruction including delaminated ciliated epithelium, interlamellar junction, and water tubes. The animals exposed to the increased concentration of TiO_2 displayed severe variations in the surface of the lamellae, structure, hyperplasia lamellae, and observed a completely disorganized mass of gill lamellae. Leite et al.[29] explain that exposure to rutile TiO_2 to marine clams *M. galloprovincialis* for 28 days causes widening of mussels' middle vessel and a profusion of lipofuscin. Whereas Stalin et al.[45] stated the protruding of principal filament gill tips, twisting of subordinate filament, gill lamellae, hyperplasia, necrotic and fusion of gill lamellae along with alterations in biological activities, such as inhalation, nutrient uptake, and ionic regulation of *L. marginalis* exposed to chlorpyrifos. Therefore, the present results correlate with other studies that previously confirmed histological variations in clam lamellae when susceptible to contaminants, specifically lanthanum[63], mercury[64,65], and 'bulk'TiO_2 and TiO_2 NPs[66].

Conclusion

This study provides novel insights into the molecular mechanisms underlying the interaction of TiO_2 nanoparticles (NPs) with the freshwater mussel *Lamellidens marginalis*. The findings highlight the potential ecological risks associated with TiO_2 NP contamination in aquatic environments, emphasizing the urgent need for stringent regulatory measures and continuous environmental monitoring. Acute exposure to environmentally relevant concentrations of TiO_2 NPs induced significant physiological alterations, including changes in the condition index, filtration rate, clearance rate, hemocyte count, metabolic capacity (electron transport system activity), and energy reserves (glycogen and protein levels). Furthermore, oxidative stress biomarkers, such as antioxidant and biotransformation enzyme activities and lipid peroxidation levels, were markedly affected. Histological analysis revealed severe gill tissue damage in mussels exposed to higher TiO_2 NP concentrations, further confirming their toxic effects. Prolonged exposure to these nanoparticles may compromise the survival, growth, population dynamics, and reproductive capacity of freshwater mussels, thereby posing a significant threat to aquatic biodiversity. While this study enhances our understanding of TiO_2 NP toxicity in freshwater invertebrates, further research is required to assess the broader ecological implications and the risks posed by other emerging contaminants in aquatic ecosystems.

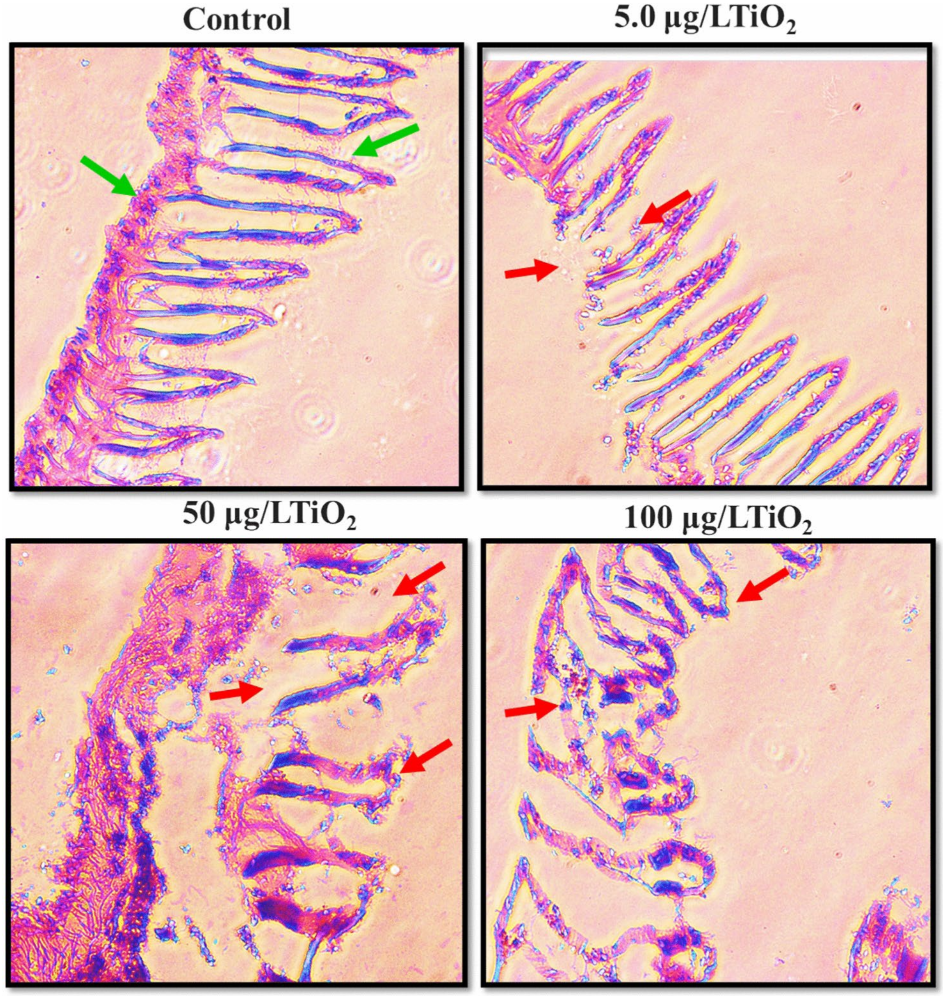

Fig. 5. Histological images of gills of *L. marginalis* exposed to TiO_2 after 7 days at each condition (CNT 5, 50 and 100 μg/L). Red arrow indicates the site of severe gill damage due to the accumulation of TiO_2 and green arrow indicates the point of less damage or no damage to the gills.

Data availability

The data supporting this study's findings are available from the corresponding author upon request.

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Author contributions

AS: methodology, data collection, formal analysis, and manuscript preparation; PV and AKN: Support in methodology, manuscript editing, and Software; CS and BV: Conceptualization, Supervision, project administration, and finalized manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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