

Toxicological impact of TiO₂ nanoparticles on *Eudrilus euginae*

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Abstract: The concern regarding the toxicological effects of nanoparticles (NPs) on the terrestrial environment is increasing. To avoid risks of exposure to these NPs in the environment, it is essential to develop an understanding of their reactivity, toxicity, and persistency. Due to the increased usage of nano-titanium dioxide (TiO₂) in various industrial products, an exponential increase in exposure is expected, which would exacerbate concerns about its ecological risks. The present study is conducted to evaluate the size-dependent effects of TiO₂ NPs on the soil, especially on earthworm (*Eudrilus euginae*). To date, many studies have been reported on the impact of TiO₂ NPs on ecotoxicology. However, histotoxicology studies are sparse. This study serves to be the first report on the size-dependent histotoxicological impact of nano-TiO₂ on earthworms particularly, *E. euginae*. This report presents an intensive overall view of the longer time ecotoxicological impact of TiO₂ nanomaterials on various biological parameters of earthworms at cellular levels. The results show that the survival and growth of adult earthworms are severely affected by the TiO₂ NPs in the soil, which substantiates the adverse effects of TiO₂ NPs on earthworms.

1 Introduction

Nowadays, nanomaterials play an important role in the manufacture of numerous products that we use in our daily life. Among them, titanium dioxide (TiO₂) is a well-known photocatalytic semiconducting material having great potential as photoelectric converters with applications in solar cells, environmental purification and for the generation of hydrogen gas [1, 2]. The vast applications of TiO₂ nanoparticles (NPs) also include various consumer goods and products of daily use, such as cosmetics, paints, dyes and varnishes, textiles, paper, plastics, and even in paving stones. So, the increased usage of these NPs in various fields has led to their presence in the soil and environment. Hence, the safety of NPs in relation to their toxicity, reactivity, and persistency, has to be analysed carefully before exposing these NPs to the terrestrial environment and ecosystem. Reports available on the effect of NPs on the natural environment could pose a threat to beneficial microbial communities such as those found in soil [1–3]. NPs can enter the soil through industrial spills, landfill sites or when sewage sludge is used as a fertiliser [4]. Due to the exceptional properties possessed by them, NPs are also able to pass through cell membranes in organisms. Reports are available on the toxic effects of TiO₂ NPs stating the formation of reactive oxygen species (ROS) with water in the presence of ultraviolet irradiation and even in the absence of photo-activation. ROS and free radicals cause oxidative stress and may play a major role in the NPs potential toxicity to organisms [5–7]. Also, the toxicity of NPs is related to their size [8]. However, the detailed interactions of these NPs with biological systems are relatively unknown [9]. Thus, leaching of NPs into the environment could be a serious problem.

In the present investigation, TiO₂ NPs with different particle sizes have been synthesised by the sol–gel method and were characterised by X-ray diffraction (XRD) and transmission electron microscopy (TEM). Our major concern is to evaluate the effect of these TiO₂ NPs at the tissue level of biological organisation to improve the understanding of hazards and risks of NPs in the environment especially on earthworm (*Eudrilus euginae*). The

effect of commercially available nano-TiO₂ on selected earthworm species was also compared and analysed. Earthworms (from the family Eudrilidae) were selected as one of the key indicator organisms for eco-toxicological testing of TiO₂ NPs. Being a soil dwelling organism, it is involved in the process of soil formation and organic matter decomposition and is widely used for vermicomposting.

2 Materials and methods

2.1 Materials

Titanium (IV)-*n*-butoxide (Ti(OBu)₄) (98%, Alfa Aesar), Ce(NO₃)₃·6H₂O (99.5%, Alfa Aesar), isopropyl alcohol (99%, Fisher Scientific) and HNO₃ (99.9%, Merck) were used for the synthesis of TiO₂. Distilled water was used in all the synthesis processes.

2.2 Synthesis of TiO₂ NPs

The procedures for the synthesis of TiO₂ NPs and Ce-doped TiO₂ NPs have been described elsewhere [10, 11]. Ce was doped to reduce particle size, and enhance visible light activity. The precursor solution was a mixture of 1:2 titanium (IV)-*n*-butoxide (Ti(OC₄H₉)₄) and isopropyl alcohol ((CH₃)₂CHOH). The required weighed % of Ce(NO₃)₃·6H₂O was dissolved in distilled water, and then mixed with another solution whose pH value was suitably adjusted to 2.0 by adding HNO₃ under a continuous stirring process. The precursor solution was then added drop wise into this solution and stirred continuously for 3 h. After complete hydrolysis of Ti(OC₄H₉)₄, and performing gelling, ageing and drying for 13–20 h, the colloid was calcined at 400°C for 3 h to obtain Ce-doped TiO₂ NPs. Ce ion-doped TiO₂ at various molar concentrations 3, 2, and 1 mol % were denoted as T1, T2, and T3, respectively. TiO₂ NPs prepared by the same method without adding Ce(NO₃)₃·6H₂O, following the calcination at 400, 600 and 800°C

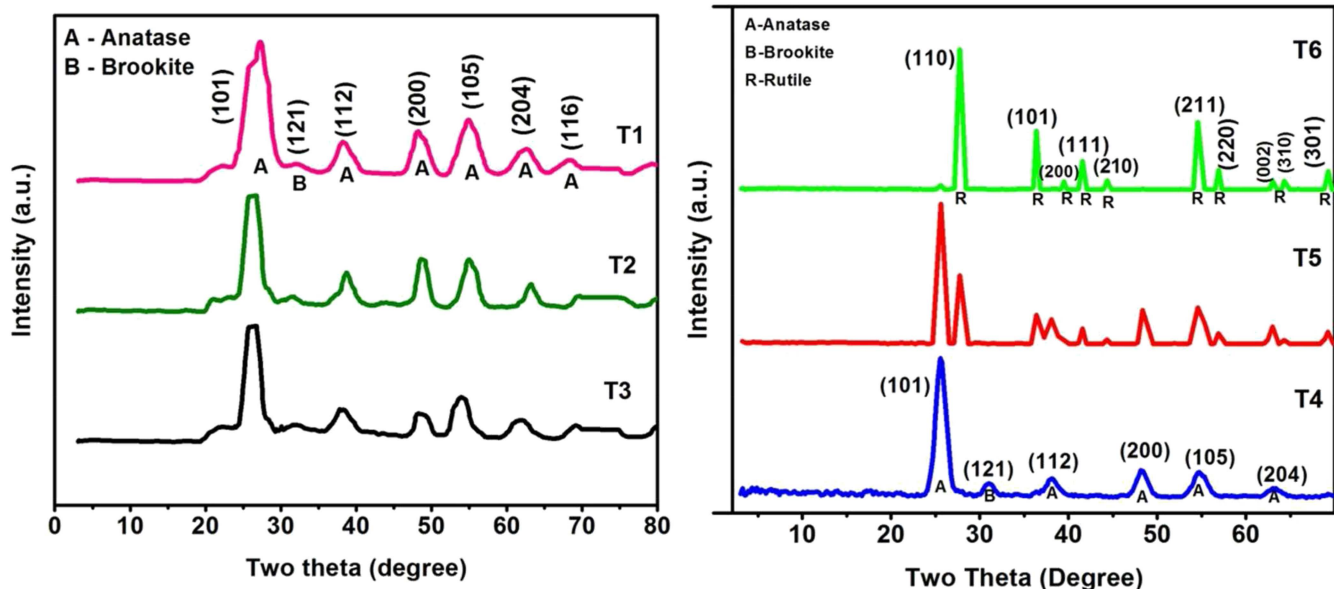


Fig. 1 XRD patterns of T1, T2, T3, T4, T5, and T6

for 3 h. TiO₂ NPs calcined at 400, 600 and 800°C were denoted as T4, T5, and T6, respectively. The order of increasing particle size is T1 < T2 < T3 < T4 < T5 < T6. Commercially available nano-TiO₂ (widely known as P25; purchased from Sigma-Aldrich) with primary particle size of 21 nm was also used to test the effect on earthworms.

X-ray powder diffraction (XRD) was carried out using a Bruker D8 Advance X-ray diffractometer ($k = 1.5406 \text{ \AA}$, step size = 0.020° , dwell time 65.6 s, 40 kV, and 35 mA) with Cu-K α_1 radiation in the 2θ range from 0° to 80° . TEM images of nanocrystalline TiO₂ powder samples were obtained using a JEOL/JEM 2100 (Source: LaB₆ and voltage: 200 kV).

2.3 Experimental set up

In the present study, exotic earthworm species *Eudrilus eugeniae* obtained from the local worm breeder associated with CARTZ, Thodupuzha, Kerala, India is used for the toxicological effect of TiO₂ NPs. The stock of culture was maintained at room temperature in a culture medium made of cow dung, grass cutting and saw dust in a ratio of 3:1:0.5. The moisture was maintained by spraying water from time to time.

The experiments were carried out in triplicates including control. To assess the effect of TiO₂ NPs on earthworms, 15 adult clitellate earthworms were introduced into the 2 kg of growth medium and admixed with 0.005% TiO₂ NPs. After four weeks, the worms were counted by wet-sieving and hand sorting. This counting has been done once in a month for six months to study the effect for a longer period.

Biological parameters, such as proliferation rate of earthworms, total heterotrophic bacterial (THB) count and fungal count, were estimated. Histology of *E. eugeniae* has been carried out by denoting the pre-clitellar region as anterior, the immediate post clitellar region as middle and the last body segments as posterior. Earthworms from each treatment were analysed according to the standard procedure [12].

3 Results and discussion

3.1 Morphology and particle size of synthesised TiO₂ NPs

Fig. 1 shows the XRD patterns of TiO₂ NP samples T1–T6. As shown in the figure, the peaks of samples T1, T2, T3, T4, T5, and T6 correspond to the tetragonal structure (space group: $I4_1/amd$) with lattice parameters $a = 3.776 \text{ \AA}$ and $c = 9.486 \text{ \AA}$, which is in agreement with JCPDS file no. 73–1764 for TiO₂. Some peaks of the rutile TiO₂ sample can also be found in T5 and T6 which is

indexed to a tetragonal structure (space group: $P4_2/mnm$) with lattice parameters $a = 4.582 \text{ \AA}$ and $c = 2.953 \text{ \AA}$, which is in agreement with JCPDS file no. 78–1510 for TiO₂.

The average sizes of crystallites, calculated by using Sherrer's equation [13–15], were 4.56, 5.2, 5.87, 6, 24 and 33 nm, respectively, for T1, T2, T3, T4, T5 and T6 samples. Sizes of the doped samples (T1–T3) are found to be decreased when compared with pure TiO₂ (T4 sample), due to the effect of Ce doping. It confirms the significant effect of Ce doping on the crystallite size of TiO₂ NPs. While for the calcined TiO₂ samples (T5 and T6), particle size increases due to thermally promoted crystallite growth occurring during calcination.

TEM images of the synthesised samples in the order of increasing particle size (T1–T6) are shown in Fig. 2. Particle size distribution for each sample is shown in Table 1 and it shows that particle sizes obtained from TEM images are in agreement with that obtained using XRD analysis.

3.2 Bio-evaluation of earthworm and media

3.2.1 Histological effects of TiO₂ NPs on earthworm: Histopathology analysis was done after 60 days exposure of NPs and studied the histological changes happening in the tested earthworms. The effect of TiO₂ NPs on the cells of *E. eugeniae* is shown in Figs. 3–5.

In control, all the layers – epidermal, circular muscle and longitudinal cell layers – are found healthy without any change (Fig. 3). However, the earthworms treated with NPs show damage in the epidermal and circular muscle layer. For the samples T1–T3 (Figs. 3 and 4), the damage is more visible in the form of shrinking of cells inside the body walls of earthworms.

The worms exposed to T4, T5 and T6 samples show more damages on the earthworm as cuticle damage, and pathological sign on epidermis, muscles, and intestinal barrier rather than T1, T2, T3 and control (Figs. 4 and 5). Maximum cell damages in the form of breakage and shrinkage of the cells are observed in the T6 sample (Fig. 5). The intensity of cell damage is in the order of T6 > T5 > T4 > T3 > T2 > T1.

In TiO₂-P25 (Fig. 6), the damage was intense with the entire epithelium being disintegrated and the epithelial cell layer was completely lost in certain regions. All these results suggest that NPs affect the histology of *E. eugeniae* leading to a negative effect on survival or reproduction depending on the type of NP exposed.

The damage of the circular and longitudinal muscle cells leads to the disruptions in the peritoneum layer of an earthworm. The disruptions in the peritoneum may lead to making problems in the functioning of blood vessels, lymph vessels, and nerves. Thus, the exposure of the test organisms to different sized NPs resulted in

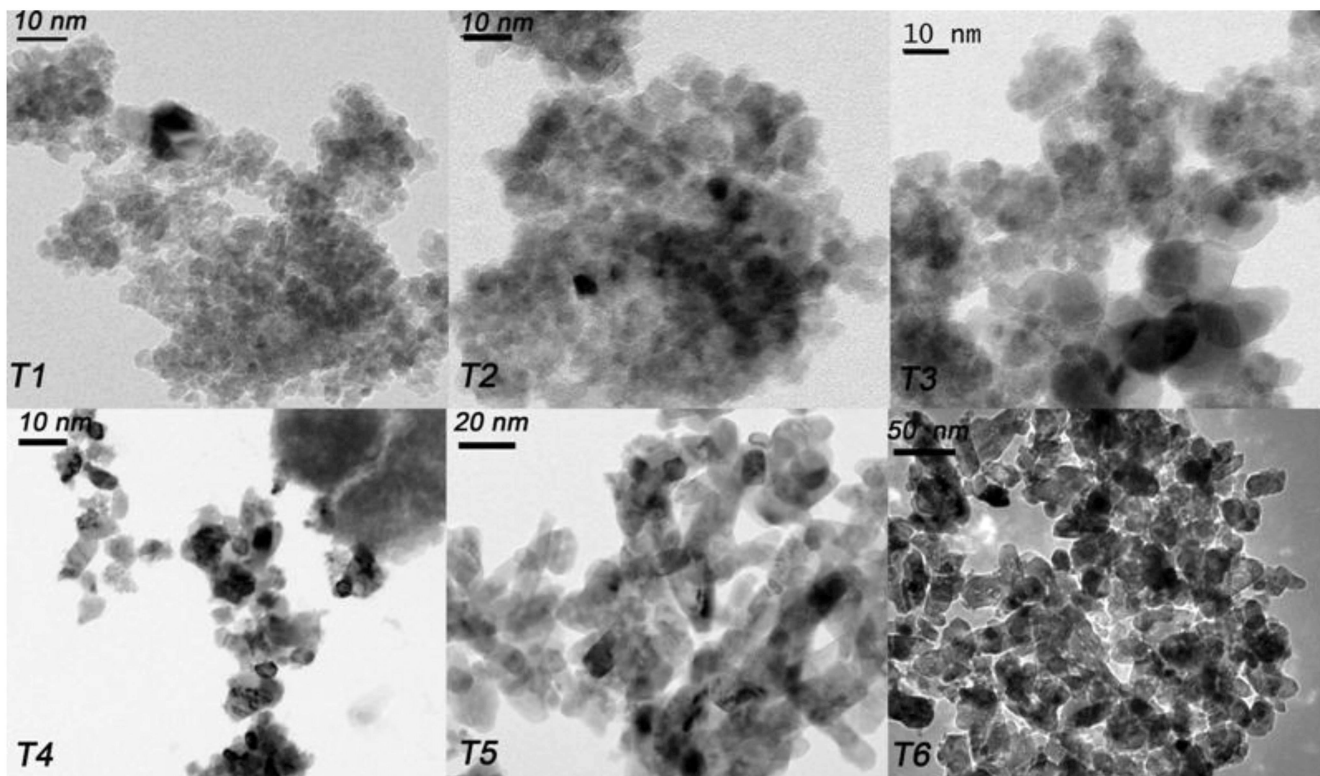


Fig. 2 TEM images of T1, T2, T3, T4, T5, and T6 TiO₂ samples

Table 1 Particle size distribution of samples T1–T6

Sample	Particle size distribution from TEM, nm	Particle size from XRD, nm
T1	5–6	4.56
T2	5–6	5.2
T3	5–8	5.87
T4	5–10	6
T5	9–35	24
T6	25–40	33

various degrees of histopathological alterations in the internal cells. The study is in agreement with the results obtained by Van Der Ploeg *et al.* [14, 15].

3.2.2 Proliferation rate and survivability of earthworms: Table 2 shows the proliferation rate and survivability of earthworms under study. It is clear that the TiO₂ NPs influence the proliferation rate of *E. euginae*. It can be seen that samples T1, T2, and T3 affected earthworm reproduction negatively, which may be due to their smaller particle sizes and presence of doped cerium ions compared with control. An increase in the proliferation rate up to 50% in samples T4, T5, and T6 were observed in comparison with T1, T2, and T3. P25 shows a slight effect on the proliferation rate. Similar results were obtained by Schlich *et al.* [16] and reported that uncoated TiO₂ NPs did not alter the rate of reproduction of earthworm (*Eisenia andrei*) in winter.

3.2.3 THB count and fungal count in media: Biological parameters like THB count and fungal count in the medium were analysed, and are presented in Fig. 7. It depicts that NP-treated samples possess maximum THB count followed by control. An increase in the fungal counts in NP-treated samples as compared with control is also observed, except for T6. Samples T1, T2, and T3 showed a decrease in proliferation rate due to the mortality of earthworms (Table 2). However, an increase in colony-forming units (CFUs) and fungal count was observed in them too. This may be due to the organic substances provided by the mortal remains of earthworms for the growth of microbes. The decrease in the fungal

count in samples T4, T5, T6, and P25, where the mortality rate of earthworms is less, implies that the NP has bactericidal and fungicidal activity [16–19]. This further concludes that TiO₂ NPs are toxic enough to kill natural bacteria and fungi present in the soil, which is shown by the reduced number of CFUs.

4 Conclusion

TiO₂ NPs are synthesised by the sol–gel method and characterised by XRD and TEM. Size-dependent toxicological impact of TiO₂ NPs on various biological parameters of *E. euginae* has been evaluated. It is found that the TiO₂ NPs exert negative effects on the earthworm proliferation rate and survivability. The percentage of mortality is found to be more with smaller TiO₂ NPs when comparing with larger particles and control. An increase in CFUs and fungal count was observed for these NPs, attributed to the decrease in the proliferation rate and increase the mortality. Histological observations made on the earthworms exposed to various TiO₂ NPs showed cuticle loss from the body wall and muscle damage in the form of breakage and shrinkage of the cells due to the formation of ROS and free radicals from TiO₂ NPs, which play a major role in the NPs' potential toxicity to organisms. These disruptions may cause problems in the functioning of blood vessels, lymph vessels and nerves of the organisms. The intensity of cell damage confirms the size dependence of TiO₂ NPs on earthworms. Moreover, all these results indicate the deleterious effect of TiO₂ NPs on the environment and eco-system.

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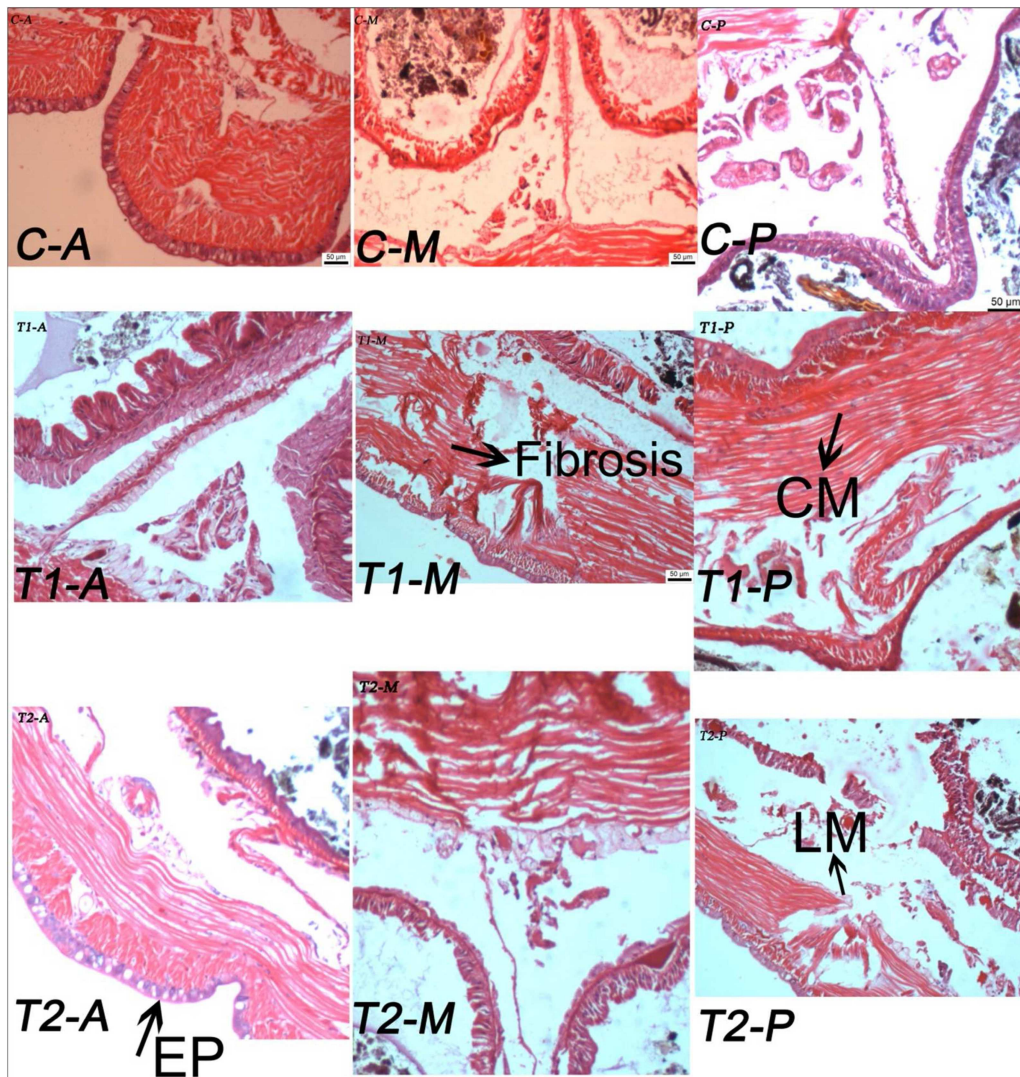


Fig. 3 Effect of TiO_2 NPs (T1 and T2) on histopathology studies of *Eudrilus eugeniae* (scale: 50 μm ; magnification 10×2048)

C: control tissue section of earth worms; T1 and T2: NP-treated tissue section of earthworm; CM: circular muscle; LM: longitudinal muscle; EP: epithelium (A, anterior; M, middle; P, posterior)

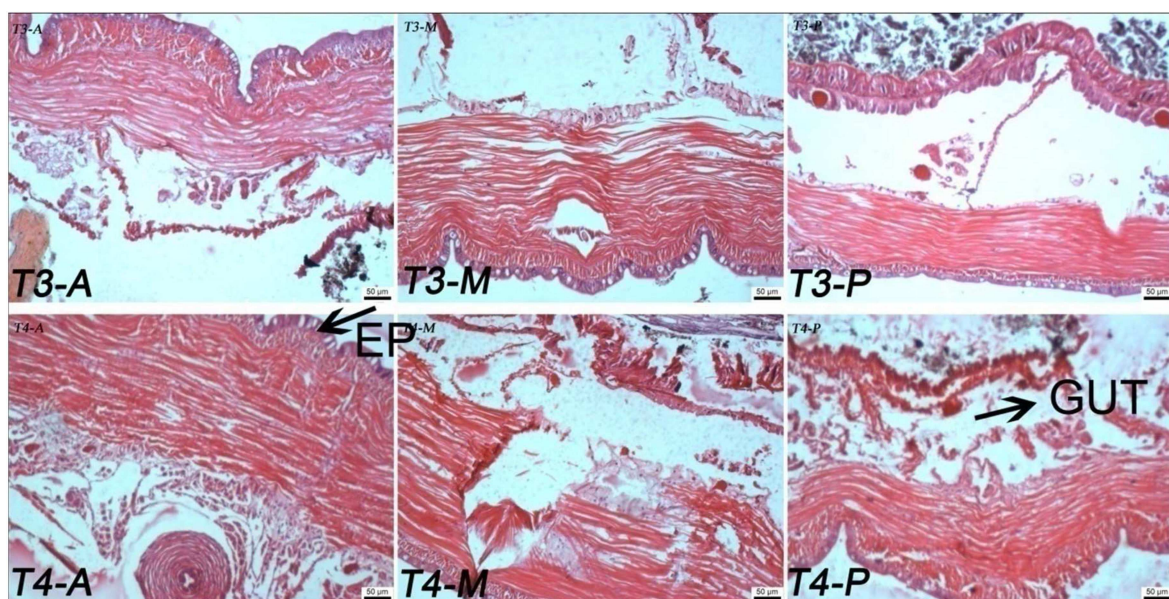


Fig. 4 Effect of TiO_2 NPs (T3 and T4) on histopathology studies of *E. eugeniae* (scale: 50 μm ; magnification 10×2048)

T3 to T4: NP-treated tissue section of earthworm; EP: epithelium (A, anterior; M, middle; P, posterior)

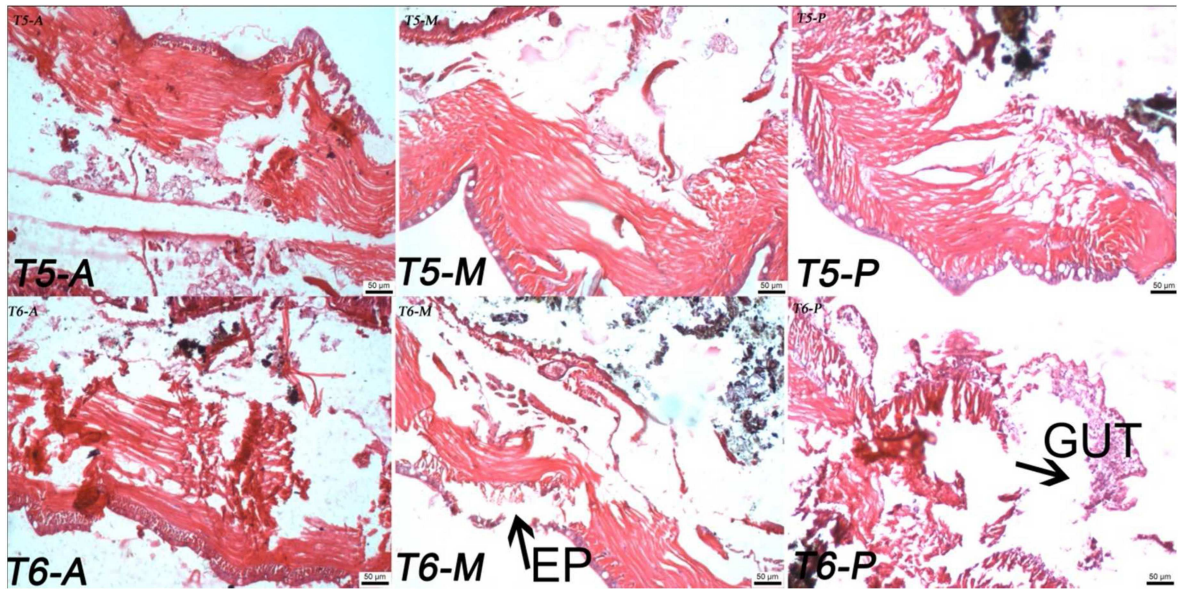


Fig. 5 Effect of TiO_2 NPs (T5 and T6) on histopathology studies of *E. euginae* (scale: $50 \mu\text{m}$; magnification 10×2048)
 T5 to T6: NP treated tissue section of earthworm; EP: epithelium (A, anterior; M, middle; P, posterior)

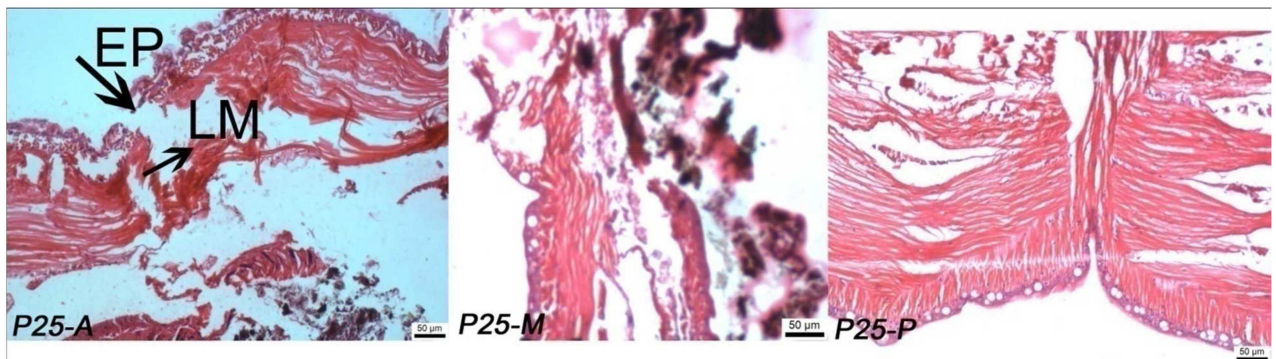


Fig. 6 Effect of TiO_2 NPs (P25) on histology studies of *E. euginae* (scale: $50 \mu\text{m}$; magnification 10×2048)
 P25: NP-treated tissue section of earthworm; EP: epithelium; LM: longitudinal muscle (A, anterior; M, middle; P, posterior)

Table 2 Proliferation rate and survivability (mean \pm SE, $n = 3$) of earthworms in samples

Samples	Initial	Final
Control	15 ± 0	59 ± 1.527
T1	15 ± 0	16 ± 2.645
T2	15 ± 0	17 ± 2.081
T3	15 ± 0	22 ± 1.0
T4	15 ± 0	54 ± 1.0
T5	15 ± 0	46 ± 0.5773
T6	15 ± 0	43 ± 1.0
P25	15 ± 0	39 ± 0.5773

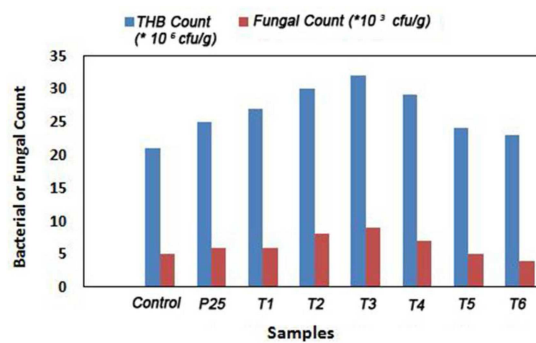


Fig. 7 THB count and fungal count

6 References

- [1] Aruoja, V., Dubourgui, H.C., Kasemets, K., *et al.*: 'Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*', *Sci. Total Environ.*, 2009, **407**, pp. 1461–1468
- [2] Blaise, C., Gagne, F., Ferard, J.F., *et al.*: 'Ecotoxicity of selected nanomaterials to aquatic organisms', *Environ. Toxicol.*, 2008, **23**, pp. 591–598
- [3] Bouche, M.B.: 'Earthworm species and ecotoxicological studies', in Greig-Smith, P.W., Becker, H., Edward, P.J., *et al.* (Eds.): '*Ecotoxicology of earthworms*' (Intercept Ltd, UK, 1992), pp. 20–35
- [4] Simonin, M., Richaume, A.: 'Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review', *Environ. Sci. Pollut. Res.*, 2015, **22**, pp. 13710–13723. doi: 10.1007/s11356-015-4171-x
- [5] Gurr, J.R., Wang, A.S.S., Chen, C.H., *et al.*: 'Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells', *Toxicology*, 2005, **213**, pp. 66–73
- [6] Reeves, J.F., Davies, S.J., Dodd, N.J., *et al.*: 'Hydroxyl radicals (-OH) are associated with titanium dioxide (TiO₂) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells', *Mutation Res.*, 2008, **640**, pp. 113–122
- [7] Armelao, L., Barreca, D., Bottaro, G., *et al.*: 'Photocatalytic and antibacterial activity of TiO₂ and Au/TiO₂ nanosystems', *Nanotechnology*, 2007, **18**, p. 375709
- [8] Chen, Z., Meng, H., Xing, G., *et al.*: 'Acute toxicological effects of copper nanoparticles in vivo', *Toxicol. Lett.*, 2006, **163**, pp. 109–120
- [9] Firdos, A.K.: '*Biotechnology fundamentals*' (Taylor & Francis, Boca Raton, USA, 2011, 2nd edn.)
- [10] Priyanka, K.P., Anu Tresa, S., Jaseentha, O.P., *et al.*: 'Cerium doped nanotitania-extended spectral response for enhanced photocatalysis', *Mater. Res. Exp.*, 2014, **1**, p. 015003
- [11] Priyanka, K.P., Sheena, P.A., Aloysius Sabu, N., *et al.*: 'Characterization of nanophas TiO₂ synthesized by sol-gel method', *Ind. J. Phys.*, 2014, **88**, pp. 657–663
- [12] Vijaya, T.M., Sushil, K.M., Usha, T., *et al.*: 'Morphological and histological studies on the vermicomposting Indian earthworm *Eudrilus eugeniae*', *World J. Zool.*, 2012, **7**, pp. 165–170
- [13] Cullity, B.D.: '*Elements of X-ray diffraction*' (Addison-Wesley, MA, 1976)
- [14] Varghese, T., Balakrishna, K.M.: '*Nanotechnology: an introduction to synthesis, characterization and applications of nanomaterials*' (Atlantic Publishers and Distributors, New Delhi, 2010)
- [15] Van Der Ploeg, M.J.C., Baveco, J.M., Van Der Hout, A., *et al.*: 'Effects of C60 nanoparticle exposure on earthworms (*Lumbricus rubellus*) and implications for population dynamics', *Environ. Pollut.*, 2011, **159**, pp. 198–203
- [16] Velhal, S.G., Kulakrni, S.D., Jaybhaye, R.G.: 'Titanium dioxide nanoparticles for control of microorganisms', *Int. J. Res. Chem. Environ.*, 2014, **4**, pp. 192–198
- [17] Ameer, A., Ahmed, A.S., Mohammad, O., *et al.*: 'Antimicrobial activity of metal oxide nanoparticles against gram-positive and gram-negative bacteria: a comparative study', *Int. J. Nanomed.*, 2012, **7**, pp. 6003–6009
- [18] Maness, P.C., Smolinski, S., Blake, D.M., *et al.*: 'Bactericidal activity of photocatalytic TiO₂ reaction: toward an understanding of its killing mechanism', *Appl. Environ. Microbiol.*, 1999, **65**, pp. 4094–4098
- [19] Schlich, K., Terytze, K., Hund-Rinke, K.: 'Effect of TiO₂ nanoparticles in the earthworm reproduction test', *Environ. Sci.*, 2012, **24**, pp. 1–10