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RESEARCH ARTICLE

EFFECT OF TITANIUM DIOXIDE NANOPARTICLES IN ENZYMATIC ACTIVITY OF EPIGEIC EARTHWORM SPECIES "*EUDRILUS EUGENIA*"

***Anitha Kumari P., and Thangam Y.**

J.K. K.Nataraja College of Arts and Science, Komarapalayam, Namakkal

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**Corresponding Author:*
Anitha Kumari, P.

ABSTRACT

Titanium dioxide nanoparticles (TiO2 NPs) are commonly used in different industries because of their physico-chemical properties. They are widely used and their environmental occurrence has raised concerns about the potential toxicity to biota as the release of nanoparticles to the soil directly or indirectly through air and water is gradually increasing. The soil is contaminated with nanoparticles in the long term and soil microorganisms can adversely be affected by these accumulated nanoparticles. Studies show that nanoparticles can have a lethal effect on soil microorganisms by causing the production of reactive oxygen species by damaging the membrane permeability, cell signaling processes, and the stability of enzymes and protein structures. This study investigated the effect of $nTiO_2$ (0, 5, 50, and 500 mg/kg) on the phenotypes, transcriptomic, and metabolomic profiles of earthworm (Eudrilus eugenia) in soil. The results showed that the antioxidant system and the transcriptomic and metabolomic profiles of earthworms were significantly affected. The superoxide dismutase (SOD) activity and the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio significantly decreased under the 500 mg/kg $nTiO_2$ treatment. The metabolomics analysis showed that glycine and pyroglutamic acid contents involved in the GSH metabolism were significantly altered under the 500 mg/kg treatment. Moreover, transcriptomics and metabolomics data revealed that the long-term exposure to $nTiO_2$ affected the synthesis of carbohydrates, proteins, and lipids. However, the transcriptomics results indicated that the genes involved in ribosome biogenesis in eukaryotes pathway and TGF-beta signaling pathway were upregulated, which could explain why the growth and reproduction of earthworms were apparently not affected by the $nTiO_2$ exposure.

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INTRODUCTION

Nanotechnology is a dynamically developing field producing large amounts of nanocompounds that are applied in industry, daily life, and health care. During production, use, and waste these materials could end up in water or soil. Large scale contaminations of our environment are a threat to public health. Pollution can have harmful effects on the immune system, as revealed by numerous studies in humans and other vertebrates. The relative simplicity of invertebrate immune functions offers potentially sensitive and accessible means of monitoring the effects and complex interactions of nanoparticles which ultimately affect host resistance. Among terrestrial and freshwater invertebrates, earthworms and leeches are the "keystone" species to evaluate the health of our ecosystems. In this review we compare the conserved stress and immune responses of these invertebrate model organisms toward nanoparticles. The obtained knowledge provides exciting insights into the conserved molecular and cellular mechanisms of nanomaterial related toxicity in invertebrates and vertebrates. Understanding the unique characteristics of engineered nanoproducts and their interactions with biological systems in our environment is essential to the safe

Nanoparticles exhibit greater reactivity as their sizes, less than 100nm size, are so small that their surface area is larger. Their transitional zone between atom or molecule and corresponding bulk material is more far from one another, which makes the original characteristics of the substance change (Hoet *et al*., 2004; Lin and Xing, 2007; Moore, 2006; Nel *et al*., 2006; Yang and Watts, 2005). It is also reported that such small size of substances can intrude into human body more easily and is more toxic than bulk material due to increased reactivity to cells in the body. Titanium dioxide nanoparticles ($nTiO_2$) are widely used and their environmental occurrence has raised concerns about the potential toxicity to biota. However, few studies have investigated the effect of long-term exposure to $nTiO_2$ on soil invertebrates. The combination of transcriptomics and metabolomics reveals the global responses that cannot be observed by conventional toxicity endpoints, facilitating the assessment of long-term ecological effect of engineered nanoparticles in the environment. TiO_2 NPs have photocatalytic properties, protect against UV radiation, are used as semiconductors, etc. These nanoparticles are used, e.g., in cosmetics, food industry, paints, ceramics, devices development, and the agriculture industry. In the last decade, TiO_2 NPs have been used in wastewater treatment plants for their ability to degrade some organic pollutants. Thus TiO_2 NPs reach the soil system from different

These nanoparticles then interact with the soil biota. It is therefore very important to assess the potential risk of TiO_2 NPs to soil organisms. DNA damage by reactive oxygen species (ROS), lung inflammation, genotoxicity, apoptosis, and inflammation are among the significant concerns that need to be taken into consideration if people are exposed to Titanium dioxide (TiO2). TiO2 nanoparticles could be synthesized in various sizes, going from 1-1000nm which is utilized for the most part in sunscreens, toothpaste, paints and coatings, agribusiness, medication and drug delivery in light of its low cost, chemical stability, antimicrobial properties and so forth. But it's also extremely important to establish that these titania incorporated materials are good for usage. Anatase (yellow to blue), rutile (deep red), and brookite (brown to black) are three predominantly occurring crystalline forms of TiO2 nanoparticles. Studies indicate that anatase is the most efficient and used form among these three. Some studies say anatase is also more cytotoxic when compared to the rutile form. However, rutile is viewed as the most favored one to be utilized in sunscreens due to its high absorbance index. Brookite is quite difficult to synthesize and is known to have a high photocatalytic activity. Among the countless beneficial properties of TiO2, the most important one is its photocatalytic activity and high refractive index. It is speculated that there are three routes through which titanium dioxide enters the human body; 1) dermal entrance by the usage of sunscreens and topical treatments 2) oral admission which is through consuming food and 3) inhalation at working environments. Once inside the body, it is phagocytized into cells, where it might bind to the mitochondria, destructing its membranes and electron transport chains or it might attach to the cell nucleus causing DNA damage and altering the gene expression, eventually stimulating loss of cell functionality. Titanium dioxide (TiO2) regardless of its various points of interest for which it is being utilized in many industries like food, paper, and cosmetics is the main concern for causing toxicity in humans, sea creatures, and the environment. In India, more than half of the population uses sunscreen on a daily basis. The affectability towards titanium is high to the point that even the individuals working in a basic printing shop are at a serious danger of TiO2 being invested in their bodies. Titanium dioxide in 2010 has also been classified as a group 2B carcinogen by the International agency for research on cancer.

Titanium dioxide is not only detrimental to humans but also has severe effects on marine life. TiO2 released into the environment is eventually getting blended with the ocean water which makes the water unfit for the organisms to live. The major release sources of this chemical into the environment are via sunscreens, health care products, effluents from industries, volatile particles from factories, anti-fouling components of paints, and from many other routes. Consequently, the sea waters are getting contaminated and therefore we are witnessing worse effects on humans and marine creatures. The concentration of TiO2 nanoparticles released from paints and coatings in the water when compared with other nanoparticles was calculated to be a number as high as 3.5 × 108 particles/L. Titanium dioxide is reported to be one of the leading causes of the generation of reactive oxygen species (ROS) such as hydroxyl radicals(OH·), hydrogen peroxide (H2O2), superoxide anions(O2-) that causes oxidative stress. Oxidative stress occurs when there is an imbalance of free radicals and antioxidants in the body. Uncontrolled oxidative stress can trigger the aging process and may accelerate tissue and DNA damage. Health conditions linked to oxidative stress are cancer, Alzheimer's disease, diabetes, chronic fatigue syndrome, cardiovascular diseases, etc. Blend of TiO2 with sunscreen lotions shows properties to reflect, scatter, and absorb UV radiations, resulting in photocatalysis and generation of ROS. Instead of preventing the oxidative stress generation by UV radiation, Titanium dioxide nanoparticles contribute to its formation. Researchers state the fact that TiO2 is a ROS generator and therefore it encourages many mutations in humans. Many studies have been conducted that measures the markers of oxidative stress in humans as well as in marine animals, for example, lipid oxidation gives rise to unstable markers of oxidative stress in the cell-like 4-hydroxy-trans-hexenal(HHE) and 4-hydroxy-trans-nonenal(HNE); that have the affinity for proteins and DNA and are considered cytotoxic.

Modulation and doping of nanoparticles are expansively being used these days since they improve their properties. Transition metals like Zn,Cu, Ag, Fe, Al, Ni, and several others are considered as dopants for nanoparticle-based semiconductors. Doping of TiO2 nanoparticles with Cu is recognized to improve its photocatalytic activity and hence is reported to be used for nanomedicine, agriculture, defense industry, and remediation of water; Although, these doped nanoparticles apparently have some side effects as well. Cu doped TiO2 nanoparticles reportedly lead to increased toxicity and ROS generation. This review summarizes the toxic effects of titanium dioxide (TiO2) nanoparticles through examinations conducted on human cell lines, human exposure to TiO2 in everyday life, and on marine organisms like sea mussels and marine algae. It additionally centers around the inception of reactive oxygen species (ROS) and reasoning that titanium dioxide should be answerable for causing oxidative stress instead of turning it away. The ecotoxicology of Nanoparticles has mostly been studied using bacteria and aquatic species. The studies to address the effects on soil dwelling invertebrates are in a preliminary stage. Preliminary results of soil species have revealed the diverse nature of the responses to the different types of NPs. Earthworms is dominant soil invertebrate animals. They possess a strong immune system because of their permanent contact with soil bacteria, viruses, and fungi. Defense mechanisms are used in earthworm protection against soil pollutants including nanoparticles. *Eudrilus eugeniae was* selected as a model organism for this study due to it being an important species in toxicity testing of soil. The objective was to study the Enzyme activity of earthworms, Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferases (GST), Superoxide dismutase (SOD), and Acetylcholinesterase (AChE) during toxicological studies.

Further, these nanoparticles can induce, e.g., oxidative stress, DNA damage, apoptosis, and affect gene expression. Earthworm cellular defense mechanisms are based on coelomocytes present in the coelomic fluid. Coelomocytes can be divided into free chloragogen cells called eleocytes, with a mainly nutritive function, and amoebocytes, which are the immune effector cells. Amoebocytes can be further divided into granular (GA) and hyaline (HA) amoebocytes. Various nanoparticles were described to impair earthworm defense mechanisms. Hayashi *et al*. showed that Ag NPs altered the expression of some genes involved in coelomocyte oxidative stress and immune reactions. TiO2 NPs cause significant mitochondrial dysfunction by increasing mitochondrial ROS levels and decreasing ATP generation in macrophages. Moreover, TiO2 NPs exposure activated inflammatory responses and attenuated macrophage phagocytic function. In earthworms, increased apoptosis was observed following TiO2 nanocomposites exposure. A compromised immune system may result in a decreased reproductive rate and increased mortality of earthworms. The study to assess the potentially dangerous impact of TiO2 NPs exposure on earthworms' cellular function, including the immune responses to harmful stimuli shows that when E. andrei coelomocytes were exposed to 1, 10, and 100 _g/mL of TiO2 NPs for 2, 6, and 24 h in vitro. After exposure, viability, oxidative stress (reactive oxygen species and malondialdehyde production), immune functions (phagocytosis), and genotoxicity (DNA damage) were assessed. Further, electron microscopy (transmission and scanning) enabled TiO2 NPs localization on the cell surface. Gene expression changes were also followed to better understand the underlying cellular mechanisms.

MATERIALS AND METHODS

Purchase of Titanium Dioxide nanoparticles: Titanium Dioxide nanoparticles were purchased from Sigma Aldrich, Chennai.

Table 1. Characteristics of TiO_2 nanoparticles

S. No	Parameters	
1	Appearance (colour)	White
2	Appearance (form)	Powder
3	Particle size	10-25
4	surface area	45-55 m^2/g

Collection and Culture of Earthworms: Earthworms *Eudrilus eugeniae* were used for the study. These earthworms were purchased from Mani Organic Farm, Salem District, Tamil Nadu, India and cultured in cement tanks for further studies. The earthworms were reared in garden soil and garden waste in a vermibed of dimension 4 x 2 x 4.4 feet (length x breadth x height) sufficient for 2,000-3,000 worms with a controlled moisture content of 60-70% and temperature between 26 and 28ºC. Nylon net was used to cover the bed to prevent the entry of predators. Adequate watering was done daily to maintain optimal moisture conditions in the vermibed.

Eudrilus eugeniae **used for vermiremediation**

The earthworm *Eudrilus eugeniae*, commonly known as the African Night Crawler (ANC) is one of the species used for vermicomposting (Guerrero *et al*., 1999). The species originates from Equatorial West Africa and is widely distributed in tropical and subtropical countries (Guerrero *et al.,*1984). The use of *E.eugeniae* in outdoor vermiculture is limited to tropical and sub-tropical regions because it prefers warmer temperatures and cannot tolerate extended periods below 16°C (Viljoen and Reinecke, 1992). It has very high rates of reproduction and is capable of decomposing large quantities of organic wastes rapidly (Edwards and Arancon, 2004).

Collection of Soil and cowdung: Top soil is collected from Periyar University garden not exceeding a depth of five inches. Then the collected soil was sun dried by spreading it on a flat, clean broad surface for 48h. The dried soil was sieved using a 2mm diameter sieve to remove debris adopted by Khan *et al*. (2012) and also was done soil spiking stainless steel spoon method (Doick *et al*. 2003). Physico-chemical characteristics of soil: pH: 7.2 ± 0.01; EC: 1.8 ± 0.05 (dsm^{-1}); OC: 6.8 ± 0.05 (%); N: 0.3 ± 0.02 (%); K: 0.1 ± 0.01 (%); P: 0.04 ± 0.01 (%); C:N Ratio: 20.3. Cow dung (CD) was collected from a Cowshed in Karuppur, Salem, Tamil Nadu, India. CD was freshly used for further experimentations and Physico-Chemical characteristics of CD: pH: 8.2 ± 0.10; EC: 0.11 ± 0.02 (dsm^{-1}); OC: 4.9 ± 0.15 (%); N: 0.3±0.01 (%); K: 0.008 ± 0.005 (%); P: 0.03 ± 0.004 (%); C:N Ratio: 15.6.

Experimental design: The earthworms were acclimatized to the laboratory conditions for a period of 60 days before the commencement of the experiment. Six circular buckets (33 cm length × 24 cm height) were used for the present study. The circular bucket was weighed with a Digital Sensitive Weighing balance (Model-CG203). The test substrates were prepared according to the ISO guidelines for earthworm toxicity testing (ISO 11268-1, 1993). The soil sample collected was sieved (≤ 5 mm) to remove coarse stones and to homogenize. 1 kg of soil was weighed into a bucket. The soil was made up21 to 60% water holding capacity using deionised water. The soil samples were contaminated with various concentrations of the TiO2 (Table-1). The mixture was thoroughly mixed manually. Furthermore, the buckets were marked as T1, T2, T3 and T4. Soil and TiO2 were taken in the proportion given in Table 2. The treatments with TiO_2-contaminated soil were left for 10 days in the laboratory exposed to the elements. After 10 days, freshly collected cow dung of about 50g for each treatment was thoroughly mixed into the bucket with TiO2-contaminated soil. Immediately after addition of additives, earthworms were sorted out from the holding containers, washed with clean water and ten earthworms of the species *Eudrilus eugeniae* contaminated soil. A netting material was placed on top of each of the containers and the cover lid frame was used to hold the containers firmly. This is done to avoid escape of the earthworms and to allow free flow of oxygen into the treatment during the course of the experiment. The setup was placed inside the laboratory and checked morning and evening on a daily basis for 21 days.

Table 2. Titanium Dioxide concentration in various vermicompost soil samples

S.NO	Treatments	Earthworms *Eudrilus eugeniae-(n)*	Soil (kg)	Cow dung(g)	TiO2 mg/kg
1	T1	10	1	50	0
2	T2	10	1	50	5
3	T3	10	1	50	50
4	T4	10	1	50	500

Analysis of Soil Samples: Physicochemical characteristics of soil like Organic carbon were determined by Partial Oxidation Method (Jackson, 1995), Total Kjeldahl nitrogen (TKN) was determined by Alkaline Permangate Method (Dewis*et al*., 1970). Available phosphorus was estimated as recommended by Anderson and Ingram, (1989) and Potassium was determined flame photometrically (Jackson, 1958). The pH of the soil was determined using pH meter (Hanna, 1968). All the above nutrients and C/N ratio were analyzed every 30 days till the end of the experiment. Total Petroleum Hydrocarbons (TPH) during the above said period was determined using Atomic Absorption Spectrophotometer (AAS) (EPA method 418.1, 1978).

Statistical analysis: All experiments were carried out in duplication. Data were expressed as Mean ± Standard Error. The statistical significance for various treatments was evaluated by one-way analysis of variance (ANOVA) using SPSS version 18 (SPSS Inc., Chicago, USA). When there was a significant difference, Tukey's honestly significantly different (HSD) multiple comparison tests were performed by fixing the significance at level $P<0.05$.

pH and EC: pH and EC of vermin compost samples were measured using pH (Elico, Model-Li 120) and EC meter (Amber science inc. Model 1056). 20 ± 0.1 g of vermicompost was added in 100 ml of distilled water and then stirred for 10 min and left for 30 min and pH was measured in supernatant liquid using pH meter. For electrical conductivity, the above mixture was left for 1 hour and then measured for conductivity using an electrical conductivity meter.

RESULTS AND DISCUSSION

Biochemical analysis: Tissue samples were homogenized in 0.1M Na-K-phosphate buffer T. Lammel, *et al*. *Aquatic Toxicology 213 (2019) 105193*(pH 7.5) by application of ultrasound pulses while being incubated in an ice-cold water bath. Subsequently, the homogenates were centrifuged at 10,000 x g for 20 min at 4 °C, and the supernatant (S9 fraction) was collected, aliquoted and stored frozen at -80 °C until biochemical analysis. Glutathione reductase (GR) activity was measured using the method described by Cribb *et al*. (1989) (Cribb *et al*., 1989).

First, samples were diluted in 0.1M sodium phosphate buffer (pH 7.5) containing EDTA (1 mM), NADPH, and DTNB. Then, the reaction was initiated by adding GSSG (concentration in the assay: 0.25 mg/ml) and the increase in absorbance was measured at 415 nm in a Spectra Max 190 microplate spectrophotometer. Glutathione S-Transferase (GST) enzyme activity was measured as described by Habig *et al*. (1974) adapted to a microplate reader as described in Stephensen *et al*. (2002) (Habig *et al*., 1974; Stephensen *et al*., 2002). In brief, samples were diluted in 0.1M sodium phosphate buffer (pH 7.5) containing 2mM CDNB and 1mM GSH, and the absorbance was measured at 350 nm in a Spectra Max 190 microplate

Table 3. Enzyme activity of earthworms, Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferases (GST), Superoxide dismutase (SOD), and Acetylcholinesterase (AChE)

Enzyme	T1	T2	T3	T4
GPx (nmol/min/mg)	11.34±0.02	11.12±0.01	10.83±0.04	10.13±0.02
GR (nmol/min/mg)	6.43±0.07	6.23±0.01	6.11±0.04	4.32±0.08
GST (µmol/min/mg)	18.94±0.04	17.12±0.05	16.93±0.07	16.12±0.06
SOD (U Misra/mg])	4.87±0.02	4.45±0.01	4.15±0.00	3.27±0.04
AChE (nmol/min/mg)	54.21±0.45	51.69±0.16	48.17±0.29	41.76±0.98

Glutathione Peroxidase (GPX) activity was assayed in a coupled reaction by detecting the changes in the NADPH level. Hydrogen peroxide and cumene hydroperoxide (Sigma, St. Louis, Mo.) were used as substrates; they were reduced by GPX with glutathione as the reducing agent. The GSSG was reduced by GR with NADPH as the substrate. The NADPH level was determined by the A340 in an LKB Ultraspec 4050. All the reagents were prewarmed to room temperature before performance of the assay. The final concentrations of these reagents in the reaction mixture were 0.5 mM peroxide (cumene hydroperoxide or hydrogen peroxide), 1 mM GSH, 0.1 U of GR, 5 mM K2HPO4, 0.2 mM EDTA, 0.2 mM NaN3, and 0.1 mM NADPH. As a negative control, bovine serum albumin was used in the reaction mixture. The enzyme activity was determined from the linear portion of the absorbance curve. One unit of GPX activity was defined as the amount of enzyme required to cause the oxidation of 1 nmol of NADPH per min under the above-described assay conditions. For each stage, at least two independent samples were assayed, and each sample was assayed at least twice, in duplicate each time. The extinction coefficient was 6,200 M21 cm21. The activity of superoxide dismutase was determined by the method described by Magwere. This involves measuring the SOD inhibition of the auto-oxidation activity of epinephrine at pH 10.2 and 30ºC. One unit of superoxide activity is defined as the amount of SOD necessary to cause 50% inhibition of epinephrine auto-oxidation. The analysis was performed in 0.02 ml of the sample and 3.0 ml of 50 M Na2CO3 buffer. This was followed by the addition of 0.03 ml of epinephrine stock solution before taking the absorbance reading at 480 nm for 3-5 minutes. A blank devoid of the sample (but containing all reagents) was used for background correction. The most widely used method for AChE activity measurement is the Ellman method (1961) based on the photometric determination of the chromogenic product coming from the reaction between acetylthiocholine (the substrate of the enzyme) and 5, 5-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent).

Statistical analysis: Statistical comparisons between multiple treatments were carried out using One Way Analysis of Variance (One-way ANOVA) followed by an appropriate posthoc test (Student-Newman-Keuls Method). When the data did not meet the requirements for the use of a parametric statistical test (i.e., normality and homoscedasticity), a non-parametric statistical test, specifically Kruskal-Wallis One Way ANOVA on Ranks was used for all pairwise comparisons. In addition, selected pairs of treatment groups were compared using a t-test or Mann-Whitney Rank Sum Test. All statistical tests were performed using SigmaPlot for Windows Version 13.0 (Systat Software, Inc.). Ecotoxicity of nanoparticles has received growing attention in recent years. This study investigated the influence of Titanium Dioxide nanoparticles (TiO_2-NPs) on earthworm *Eudrilus eugenia*. The experiment was performed with four test groups: T1 (0 mg kg^{-1}), T2(5 mg kg^{-1}), T3(50 mg kg^{-1}) & T4 (500 mg kg^{-1}). After 14-day acute exposure, activities of various enzymes, Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferases (GST), Superoxide dismutase (SOD), and Acetylcholinesterase (AChE) were determined. Data showed that the activity of GR was significantly lower at 500 mg kg^{-1}. The activities of Glutathione S-transferases (GST), Superoxide dismutase (SOD), and Acetylcholinesterase (AChE) were inhibited following the increase of TiO_2 concentration.

Data indicate that TiO2 nanoparticles may be harmful to the earthworm *E. Eugenia* at 500 mg $kg^{-1.}$ (Table 3). The activity of Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferases (GST), Superoxide dismutase (SOD), and Acetylcholinesterase (AChE) are reduced as the concentration of the Titanium Dioxide nanoparticles increases in the substrate. The activity of these enzymes are very low in the T4 sample as the concentration is comparatively very high in that sample (500 mg kg^{-1}). The effect of Titanium Dioxide nanoparticles in the enzyme activity in Earthworm *Eudrilus eugenia is* demonstrated through the figures1 to 5. Some recent studies that have examined the toxicity of nanosized TiO2 to earthworms showed that earthworms expressed toxic responses to nanosized TiO2 at very high concentrations in soil. These concentrations exceed predicted maximum TiO2 concentrations of 0.3 mg/kg in soil and up to 523 mg/kg in sewage treatment plant sludge, so it has been suggested that TiO2 nanomaterials may be nontoxic to earthworms at environmentally relevant concentrations. However, Lapied *et al*.noted increased apoptotic frequency in the cuticle and intestinal epithelium of earthworms after seven days of exposure to 100 mg/L nanosized TiO2 in water, and a similar trend was noted in earthworms exposed to only 15 mg/kg nanosized TiO2 in soil after four weeks of exposure. Therefore, the toxicity of TiO2 nanomaterials to earthworms following chronic exposure to low, environmentally relevant concentrations remains uncertain.

To further investigate the response of earthworms to chronic sublethal exposures to TiO2 nanomaterials, in the current study earthworms were raised from juveniles for between 20 and 23 weeks in soils with either 20 or 200 mg/kg of nanosized TiO2. To distinguish responses specific to particle size, this experiment also included soil treatments spiked with TiO2 with a larger mean particle size (>100 nm) at the same concentrations (20 and 200 mg/kg) in addition to an unspiked control soil. Assessment of earthworm responses to the TiO2- treated and control soil exposures was performed using metabolomics, which seeks to identify alterations in the profile of endogenous metabolites within a cell, tissue, organ, or organism following exposure to an external stressor. This technique has previously been demonstrated to provide a powerful tool for evaluating the sublethal toxicity of a wide variety of environmental contaminants to earthworms and has also been applied to identify various mechanisms of toxicity of nanosized TiO2 in rats. Since the toxic mode of action (MOA) is currently poorly understood for nanomaterials, the precise physiological parameters that should be monitored to detect sublethal toxicity are not known. Therefore, metabolomics is well suited for the preliminary assessment of the biological response of earthworms to nanosized TiO2, since it provides a nonspecific assessment of the end result of multiple simultaneous biological processes. The use of a biomarker in biomonitoring requires the knowledge of the relationships between chemical exposure, biomarker responses and adverse effects. These aspects have been well established in the case of AChE. Several studies reported significant relationship between exposure to organophosphorus compounds and AChE inhibition in exposed worker populations . As regards the relationship between AChE inhibition and health negative effects, it is known that an inhibition of AChE between 50% and 60% elicits a dose-response pattern of relatively mild symptoms such as weakness, headache, dizziness, nausea, and salivation with a convalescence of 1–3 days. An inhibition of AChE between 60 and 90% produces moderate symptoms such as sweating, vomiting, diarrhoea, tremors, disturbed gait, pain in chest, and cyanosis of the mucous membranes which reverse within few weeks.

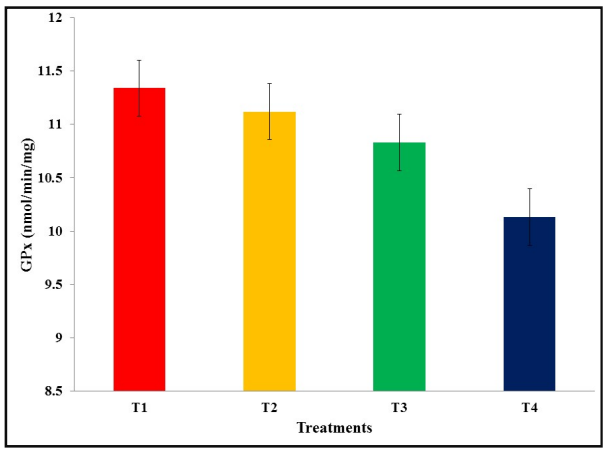

Fig. 1. Earthworm enzyme activity – Glutathione peroxidase (GPx)

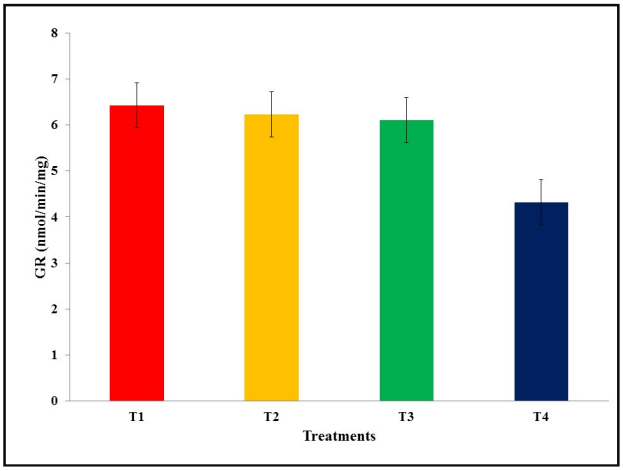

Fig. 2. Earthworm enzyme activity – Glutathione reductase (GR)

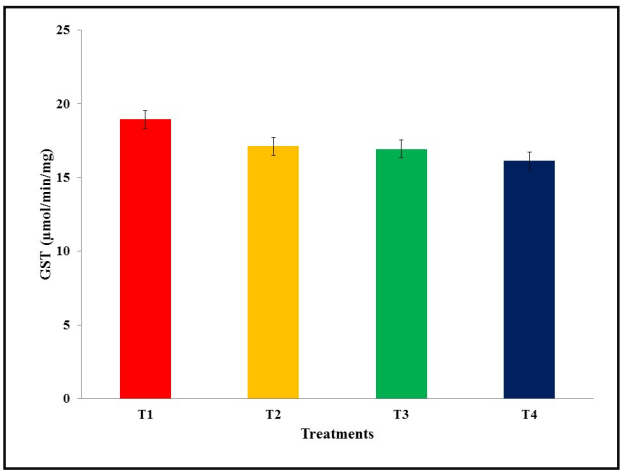

Fig. 3. Earthworm enzyme activity – Glutathione S-transferases (GST)

At 90–100% inhibition, death from respiratory or cardiac failure occurs. In the last years, the inhibition of AChE from several chemical species other than organophosphate and carbamate pesticides including heavy metals, other pesticides, polycyclic aromatic hydrocarbons, detergents, and components of complex mixtures of contaminants has been increasingly reported in humans and other animals. The potential of some metallic ions to depress the

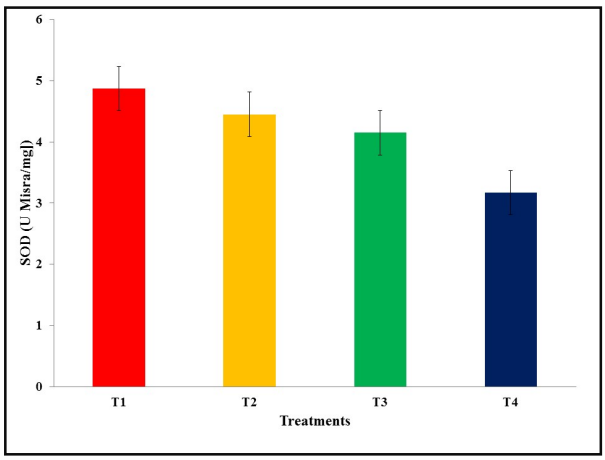

Fig. 4. Earthworm enzyme activity – Superoxide dismutase (SOD)

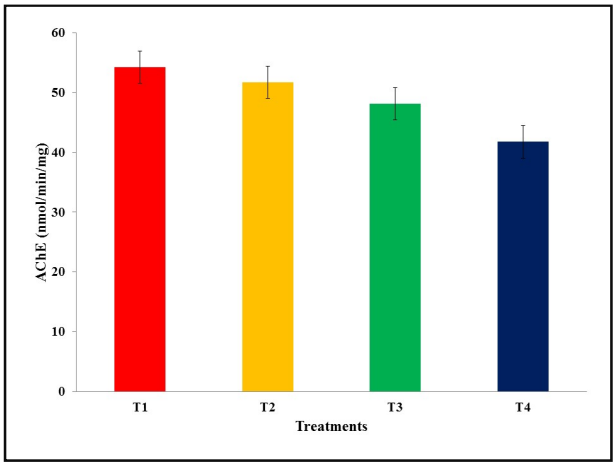

Fig. 5. Earthworm enzyme activity – Acetylcholinesterase (AChE)

demonstrated in several studies on humans and animals. Ademuyiwa *et al*. studied the potential effect of lead on erythrocyte AChE activity during occupational exposure to this metal and suggested that erythrocyte AChE activity could be used as a biomarker of lead-induced neurotoxicity in occupational exposed subjects. Recently, due to the growing interest in nanomaterials in various applications (e.g., electronics, biomedicine, catalysis, and material science), Wang *et al*. explored the potential effects of nanoparticles on AChE activity *in vitro*. Different classes of nanoparticles, including metals, oxides, and carbon nanotubes (SiO_2, TiO_2, Al_2O_3, Al, Cu, carbon-coated copper, multiwalled carbon nanotubes, and single-walled carbon nanotubes), showed high affinity for AChE. Cu, Cu–C, multiwalled carbon nanotubes, and single-walled carbon nanotubes MWCNT, SWCNT showed a dose–response inhibition of AChE activity with IC50 values of 4, 17, 156, and 96 mgL^{-1}, respectively. The inhibition by nanoparticles was primarily caused by adsorption or interaction with the enzyme. GPX may play a more important role in the protection of schistosomes because H2O2 has been shown to cause more damage to schistosomes than O2. GSTs are a family of cytosolic and membrane-associated dimeric isoenzymes that conjugate with xenobiotics for detoxification. They are related to GPX because they can function as antioxidants to reduce lipid hydroperoxide but not hydrogen peroxide . They lack selenium in the active site. Hu *et al*. (2010) observed toxicity effect exposed TiO2 nanoparticles to earthworms. They found significant DNA damage as result of comet assay to earthworms when doses were in high concentration. Bioaccumulation of Ti in earthworm was increased exposure nanoparticle concentration dependent. Qi (2009) performed acute (filter paper and sand test) and chronic (artificial soil and sandmature) toxicity with TiO2 nanoparticles. Bioaccumulation increased when

The disparity between the free radicals and antioxidants in the body promotes oxidative stress. This irregular number allows the free radicals to act together with other molecules and persuade chemical reactions well-known as oxidation reactions which could be beneficial or harmful. There could be such a significant number of reasons for increased oxidative stress in the body such as exercises, inflammation, or some environmental conditions. In the recent scenario, titanium dioxide-based nanomaterials have picked up swiftness in about every business starting from cosmetics to the textile and many more. With the increased application of titanium dioxide in sunscreens, lotions, toothpaste, and in many medicines, individuals have become more prone to their toxic effects in which the major concern is the generation of damaging oxidants. These oxidants include hydrogen peroxide, superoxide anions, and hydroxyl radicals, collectively known as reactive oxygen species (ROS). All the organisms have their own antioxidant mechanisms such as low molecular weight antioxidant molecules like Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferases (GST), Superoxide dismutase (SOD), and Acetylcholinesterase (AChE). GSH works by destroying H2O2 and SOD destroying superoxide radicals. Oxidative damage to the essential biomolecules results in alterations in some biological functions such as signal transduction and gene expression for mitogenesis and mutagenesis. As the size and surface area of TiO2 nanoparticles show greater affinity to produce reactive oxygen species, it could be anticipated from the studies that oxidative stress pathway has a role in injuries induced by nano TiO2. Titanium dioxide nanoparticles (n-TiO2) are among the man-made nanomaterials that are predicted to be found at high concentrations in the aquatic environment. There, they likely co-exist with other chemical pollutants.

CONCLUSION

Nanotechnology is becoming an attractive discipline that draws many scientists, researchers, and engineers to explore, investigate, and create many innovations. These nanomaterials have some superior physicochemical properties than the bulk materials due to their nanoscale size. This small size is critical for the enhanced physical phenomena leading to different properties in chemical and biological reactions. As the advanced exploration of nanotechnology continues, a great number of consumer products containing nanoparticles have reached the markets. The nanoparticles from sunscreen, toothpaste, detergents, and other products are finally entering sewage systems. These nanoparticles exhibit antibacterial properties, disrupt microbial activities in activated sludge, and affect the efficiency of wastewater treatment plants. Among the many nanoparticles, titanium dioxide (TiO2) is one of the most widely used nanomaterials and is present in many personal products. The TiO2 nanoparticles may exert a negative impact on aquatic ecosystems, which are related to human health. We are consistently being exposed to nanomaterials in direct and/or indirect route as they are used in almost all the sectors in our life. Nations across the worlds are now trying to put global regulation policy on nanomaterials. Sometimes, they are reported to be more toxic than the corresponding ion and micromaterials. Therefore, safety research of nanoparticles has huge implications on a national economics. In this study, we evaluated and analyzed the research trend of ecotoxicity of nanoparticles in soil environment. Test species was earthworm, *Eudrilus eugenia*. Soil enzyme activities were also discussed. It is found that the results of nanotoxicity studies were affected by many factors such as physicochemical properties, size, dispersion method and test medium of nanoparticle, which should be considered when conducting toxicity researches.

In particular, more researches on the effect of physicochemical properties and fate of nanoparticles on toxicity effect should be conducted consistently. This study investigated the effect of $nTiO_2$ (0, 5, 50, and 500 mg/kg) on the phenotypes, transcriptomic, and metabolomic profiles of earthworm (*Eudrilus eugenia*) in soil. The results showed that the antioxidant system and the transcriptomic and metabolomic profiles of earthworms were significantly affected. The superoxide dismutase (SOD) activity and the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio significantly decreased under the 500 mg/kg $nTiO_2$ treatment. The metabolomics analysis showed that glycine and pyroglutamic acid contents involved in the GSH metabolism were significantly altered under the 500 mg/kg treatment. Moreover, transcriptomics and metabolomics data revealed that the long-term exposure to $nTiO_2$ affected the synthesis of carbohydrates, proteins, and lipids. However, the transcriptomics results indicated that the genes involved in ribosome biogenesis in eukaryotes pathway and TGF-beta signaling pathway were upregulated, which could explain why the growth and reproduction of earthworms were apparently not affected by the $nTiO_2$ exposure. To understand the current research trends of nanoparticle ecotoxicity in soil environment, research papers on nanotoxicity for soil biota including plant, earthworm, soil nematode were thoroughly analyzed and discussed in terms of the kinds of nanoparticles, test species, and exposure medium. We also included soil enzyme activity. The researches demonstrated the wide range of results from one another even when using the same a nanoparticles because the particle size, surface coating, dispersion methods, and test medium supposedly made difference in the results. Therefore, future researches should be conducted by considering these factors. In particular, as soil media clearly affect their physico- chemical properties and fate of nanoparticles, future researches needs to be done in this regard.

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