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*Nanomaterials in the Environment*

# REPRODUCTIVE AND BEHAVIORAL RESPONSES OF EARTHWORMS EXPOSED TO NANO-SIZED TITANIUM DIOXIDE IN SOIL

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**Abstract**—Nanometer-sized titanium dioxide (nano-$TiO_2$) is found in a number of commercial products; however, its effects on soil biota are largely unknown. In the present study, earthworms (*Eisenia andrei* and *Eisenia fetida*) were exposed to three types of commercially available, uncoated $TiO_2$ nanomaterials with nominal diameters of 5, 10, and 21 nm. Nanomaterials were characterized for particle size, agglomeration, surface charge, chemical composition, and purity. Standard lethality, reproduction, and avoidance tests, as well as a juvenile growth test, were conducted in artificial soil or field soil amended with nano-$TiO_2$ by two methods, liquid dispersion and dry powder mixing. All studies included a micrometer-sized $TiO_2$ control. Exposure to field and artificial soil containing between 200 and 10,000 mg nano-$TiO_2$ per kilogram of dry soil (mg/kg) had no significant effect ($p > 0.05$) on juvenile survival and growth, adult earthworm survival, cocoon production, cocoon viability, or total number of juveniles hatched from these cocoons. However, earthworms avoided artificial soils amended with nano-$TiO_2$. The lowest concentration at which avoidance was observed was between 1,000 and 5,000 mg nano-$TiO_2$ per kilogram of soil, depending on the $TiO_2$ nanomaterial applied. Furthermore, earthworms differentiated between soils amended with 10,000 mg/kg nano-$TiO_2$ and micrometer-sized $TiO_2$. A positive relationship between earthworm avoidance and $TiO_2$ specific surface area was observed, but the relationship between avoidance and primary particle size was not determined because of the agglomeration and aggregation of nano-$TiO_2$ materials. Biological mechanisms that may explain earthworm avoidance of nano-$TiO_2$ are discussed. Results of the present study indicate that earthworms can detect nano-$TiO_2$ in soil, although exposure has no apparent effect on survival or standard reproductive parameters. Environ. Toxicol. Chem. 2012;31:184–193. © 2011 SETAC

**Keywords**—Earthworm Ecotoxicology Nanoparticle Soil Titanium dioxide

## INTRODUCTION

The ecotoxicological risk of engineered nanomaterials in terrestrial ecosystems is not well understood [1–3]. Some of the highest concentrations of nanomaterials will likely be found in agricultural soils receiving sewage-based solid waste, with nanometer-sized titanium dioxide (nano-$TiO_2$) predicted to be the most abundant [4]. In 2008, the annual global production of nano-$TiO_2$ was estimated at 5,000 tons [5], with use in commercial products as diverse as sunscreens, cosmetics, and antibacterial sprays [3]. The gradual replacement of micrometer-sized $TiO_2$ considered safe for human consumption (http://www.fda.gov/forindustry/coloradditives/coloradditive inventories/ucm115641.htm), with nano-$TiO_2$ in these and other commercial products, could result in $2.5 \times 10^6$ tons of nano-$TiO_2$ produced annually in the United States by 2025 [6].

The effects of nano-$TiO_2$ on organisms in complex natural media such as soils, sediments, and natural waters are poorly understood, although various components in these environments may react with nano-$TiO_2$ surfaces and thereby modify the effects [7,8]. Most in vivo research on the effect of nano-$TiO_2$ has focused on aquatic organisms [1]; to date, only two peer-reviewed articles have been published on the responses of earthworms exposed to nano-$TiO_2$ in soils. No significant mortality was reported for earthworms (*Eisenia fetida* Savigny) exposed to 5,000 mg of nano-$TiO_2$ in artificial soil for 7 d, despite increased activities in enzymes associated with oxidative stress [9]. However, the total number of juveniles produced by *E. fetida* was significantly reduced when earthworms were exposed to 1,000 mg nano-$TiO_2$ per kilogram soil for 28 d [10]. Responses of earthworms to other metal and metal oxide nanomaterials revealed no nano-specific effects on survival or the reproduction of earthworms exposed to soils amended with 1,000 mg/kg Ni, $Al_2O_3$, or $ZrO_2$ nanomaterials [10]. However, earthworm reproduction was affected following exposure to soils amended with 1,000 mg/kg Ag, and Cu nanomaterials [10] and to soils amended with $\geq 3{,}000$ mg/kg $Al_2O_3$ [11]. Whether this was because of toxicity from exposure to metal ions released through nanomaterial dissolution or from the nanoparticles themselves was not clear [10].

Earthworm movement away from a contaminated area is termed avoidance, a rapid behavioral response that can prevent injury caused by exposure to potentially damaging substances [12]. Depending on the contaminant, this response may be a more sensitive indication of harmful conditions than survival or reproduction tests [13,14]. When given a choice between unamended soil and soil amended with nanomaterials, earthworms avoided soils amended with $\geq 9$ mg/kg of Ag nanomaterials [15] as well as soils amended with $> 5{,}000$ mg/kg nano-$Al_2O_3$ [11]. The mechanisms resulting in earthworm avoidance responses, which appeared to be related to the nanomaterials themselves rather than their dissolution products, are not yet known.

The mechanisms by which nanomaterials may exert toxicity in organisms are not yet fully understood, and the same species

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may respond inconsistently to similar nanomaterials [16,17]. The disparity in results could be because of differences in methods of nanomaterial preparation and addition to media or organism exposure conditions between studies, as well as interactions between nanomaterials and solution-phase cations or dissolved organic matter in complex natural media [7,18]. Nanoparticle size, agglomeration, surface area, and surface chemistry may also modify the effects of nanomaterials on biota. For this reason, responses of standard toxicity test organisms such as earthworms to well-characterized nanomaterials using existing standard protocols will help to establish baseline nanoecotoxicological data.

However, the standard exposure metrics (mass, concentration) might not be relevant with nanomaterials, because their toxicity may be related to physicochemical characteristics such as particle size, nanomaterial specific surface area (SSA), number of particles, or particle reactivity [19,20]. As physical measurements of commercially available nanomaterials differed from values reported by the manufacturers in several studies, at least a minimal characterization of nanomaterials is a necessary part of any nanoecotoxicological test [18].

The hypothesis for the present study is that exposure to nano-$TiO_2$ in soil causes concentration-dependent effects on earthworm life cycle parameters and behaviors that are not observed during exposure to micrometer-sized $TiO_2$. The standard earthworm test species *E. fetida* and *Eisenia andrei* (Bouché) were exposed to three characterized nanomaterials in field and artificial soils using standard protocols. The nanomaterials were commercially available nano-$TiO_2$, covering a range of particle sizes and SSAs. Uncoated nanomaterials were selected to avoid effects from surface treatments. Aeroxide® P25 from Evonik Degussa was included because it has been evaluated repeatedly in the peer-reviewed literature [8,10,13]. In the absence of a standard method to add nanomaterials to soils, two methods, liquid dispersion and dry powder mixing, were tested.

## MATERIALS AND METHODS

### *Chemicals*

Chemicals used in the present study were analytical-grade NaOH, HCl, and NaCl (Fisher Scientific); $CaCO_3$ and carbendazim (Sigma-Aldrich); and boric acid (EMD Chemicals); trace metal-grade $HNO_3$ (Caledon Labs); and U.S. Pharmacopeia-grade KCl (Fisher Scientific). Ultrapure water (Millipore type; 18 MΩ/cm) was used in all experiments unless otherwise indicated. All nonmetallic equipment was presoaked in 10% HCl for more than 30 min and rinsed thoroughly in ultrapure water before use. Equipment for trace metal analysis was subsequently immersed in 10% $HNO_3$ overnight and then rinsed in ultrapure water.

### *Nanomaterial characterization*

Three uncapped, nonfunctionalized nano-$TiO_2$ materials (that is, with no surface treatments) were selected to represent a range of nano-$TiO_2$ particle sizes and SSAs (Table 1). The three test materials were material A (nominal particle size 5 nm, 100% anatase; Nanostructured and Amorphous Materials), material B (nominal particle size 10 nm, 100% anatase; Hombikat UV100; Sachtleben Chemie), and material C (nominal particle size 21 nm, 80% anatase, 20% rutile; Aeroxide P25; Evonik Industries). Two micrometer-sized test materials were used as $TiO_2$ controls: material D (nominal particle size 300 nm, 100% anatase; Hombitan LW-S; Sachtleben Chemie) and material E (nominal particle size <45 μm, 100% anatase; titanium IV oxide-325 mesh; Sigma-Aldrich).

Particle size was measured by sonicating materials in methanol for 5 to 10 s prior to evaporating a few drops on a carbon/formvar-coated copper grid and imaging by transmission electron microscopy using a model JEM-2100F field emission electron microscope set at 200 kV (JEOL Canada). Mean particle size was the diameter of at least 40 particles in the transmission electron microscopic images, determined in ImageJ software (ImageJ Ver 1.43, http://rsbweb.nih.gov/ij/). Particle crystallinity was evaluated by X-ray diffraction scanning from 10 to 100° 2-theta, using a Cu anode at a K-alpha of 1.54060 (Philips PW 1710 Reflection Diffractometer; Panalytical). The agglomerate hydrated diameter and the point of zero charge values were determined by dynamic light scattering (Malvern Zetasizer; Malvern Instruments) in aqueous suspensions, using optimal $TiO_2$ concentrations as determined by the apparatus (40 g/L for nanomaterials, 10 g/L for micrometer-sized materials). The point of zero charge values were estimated by adjusting the dispersion pH with 0.05 N NaOH or 0.05 N HCl and plotting the zeta potential at pH values ranging from 2.8 to 10.0. Agglomerate hydrated diameters were measured in dispersions with a pH value between 6.6 and 6.8. The SSA was determined by the method described by Brunauer, Emmett, and Teller (BET method) [21] with a Tristar 3000 V6.07A (Micromeritics Instrument). Metal contaminant levels were measured by inductively coupled plasma–mass spectrometry analysis (Varian 820MS) following hot $HNO_3$ digestion [22].

Table 1. Selected characteristics of titanium dioxide materials used in the present study

| Material | Mineral phase[a] | Nominal particle size (nm)[b] | TEM imaged particle size (nm)[c] | Specific surface area ($m^2$/g)[d] | Average agglomerate hydrated diameter (nm)[e] | Smallest agglomerate hydrated diameter (nm)[f] | Point of zero charge (pH)[f] |
|---|---|---|---|---|---|---|---|
| A | 100% Anatase | 5 | 20 (±7) | 141 | 829 (pH 6.8) 496 (pH 10.0) | 534 (pH 6.8) 120 (pH 10.0) | 6.20 |
| B | 100% Anatase | 10 | Not discernable | 274 | 805 | 601 | 6.30 |
| C | 83% Anatase 17% rutile | 21 | 19 (±4) | 49 | 1,209 | 867 | 6.32 |
| D | 100% Anatase | 300 | 119 (±46) | 10 | 258 | 152 | <3.50 |
| E | 100% Anatase | <45,000 | 118 (±38) | 9 | 298 | 181 | <3.50 |

[a] Measured by powder X-ray diffraction.
[b] Nominal size is the particle diameter reported by the manufacturer.
[c] Transmission electron microscopy (TEM)-imaged size was estimated by counting 40 to 50 particles in four to six TEM images. Numbers in parentheses are standard deviations. Samples were sonicated in methanol for 1 s prior to imaging.
[d] Specific surface area was measured on the powders (as delivered) using BET analysis.
[e] Measured using dynamic light scattering. Number average values are reported. Samples (40 mg/L for nanomaterials or 10 mg/L for micrometer-sized materials) were vortexed for 3 min and then measured at pH 6.6 to 6.8 unless specified.
[f] Measured using dynamic light scattering. Samples (dispersion concentrations described above) were vortexed for 3 min before measurement.

*Soil preparation*

Agricultural soil (Typic Hapludalf sandy loam of the Chicot series) was collected from an alfalfa field at the Macdonald Campus farm (Ste.-Anne-de-Bellevue, Quebec, Canada; 45°30′N, 73°35′W). Analysis of the field soil indicated a pH of 6.7 (1:2 weight/volume [w/v] soil:water slurry), with 5% soil organic matter (loss on ignition at 360°C) and water content of 50% (weight of water/weight of dry soil) at saturation. After air drying, the soil was sieved to pass through a 2-mm mesh screen and stored at room temperature (20°C). The Organisation for Economic Co-operation and Development standard artificial soil [23] was composed of 70% silica sand (90% particles having a diameter of less than 40 μm), 20% kaolin clay, and 10% peat sieved to 2 mm, and had a water content of 54% (w/w) at saturation. The pH of the artificial soil (1:2 w/v soil:water slurry) was adjusted to be between pH 6.5 and 6.7 using $CaCO_3$. Soil Ti concentration was measured by X-ray fluorescence using a fused bead preparation (Philips PW2440 4-kW X-ray fluorescence spectrometer and rhodium tube; Panalytical).

Two methods to amend soil with $TiO_2$ were tested (Table 2). In the first method, $TiO_2$ was added as a liquid dispersion (referred to as the dispersion method), whereas in the second method, dry $TiO_2$ powder was mixed with air-dry soil (referred to as the dry mix method). The dispersion method was used in survival and reproduction tests in which the maximum concentration of $TiO_2$ was 200 mg $TiO_2$ per kilogram of soil, whereas the dry-mix method was used for all tests that included concentrations of higher than 200 mg/kg $TiO_2$. Method selection was based on physical limitations, because the large volume of nanomaterials required to create the dispersions made this method unworkable when nominal concentrations $\geq$1,000 mg $TiO_2$ per kilogram of soil were required.

*Dispersion method.* A stock $TiO_2$ dispersion was created by adding 250 mg $TiO_2$ to 250 ml water in a polypropylene container and raising the pH to 10 with 0.05 N NaOH (<5 ml added). The dispersion was vortexed for 3 min and left to stabilize at 20°C for 4 h. The Na concentration in the micrometer-sized $TiO_2$ dispersion was augmented with 0.05 M NaCl solution to equal that in the nano-$TiO_2$ dispersion. Total Na concentration in soil amended with dispersion solutions ($\leq$0.084 mM $Na^+$ per kilogram soil) was below the level shown to affect reproduction and survival in *Eisenia* species [24]. After stabilization, the stock solution was revortexed and subsamples were pipetted into polypropylene containers of water to yield dispersions with nominal concentrations of 60 and 600 mg/L $TiO_2$. These were added to 500 g air-dry soil in 1-L glass jars for the acute toxicity and reproduction tests or to 100 g air-dry soil in 500-ml glass jars for the juvenile growth test. The final nominal $TiO_2$ concentration in the soil was 20 or 200 mg $TiO_2$ per kilogram of soil, and soil water content was 50 ± 1% (standard deviation [SD]) of the saturated water content. For the negative control treatment, the pH of the water used to moisten the soil was increased to pH 10 with 0.05 N NaOH, and the Na concentration was adjusted with 0.05 M NaCl to equal that of the $TiO_2$ treatments. Jars containing moist soils were closed with perforated metal lids, wrapped in aluminum foil to exclude light, and stabilized for 24 h at 20°C prior to adding earthworms.

*Dry mix method.* Between 0.1 g and 10 g of $TiO_2$ powder (micrometer or nanometer-sized) was added to 500- to 1,000-g batches of air-dry soil, placed in a polypropylene container, and mixed on a rotary shaker (Gilson Company, Lewis Center) at 60 rpm for 20 to 24 h. The quantities of soil and $TiO_2$ powder depended on the nominal $TiO_2$ concentration tested (between 100 and 10,000 mg/kg of $TiO_2$; see Table 2). The unamended negative control soil was also mixed on the rotary shaker prior to use. For the reproduction tests, 500 g mixed soil, either amended or unamended, was then weighed into a 1-L glass jar and water added to reach 50 ± 1% of the saturated water content. Soils were stabilized for 24 h at 20°C, as described for the dispersion method above. For the avoidance tests, between 2.0 kg and 4.0 kg of mixed soil was placed in a plastic bucket, thoroughly hand mixed with sufficient water to reach 50 ± 1% of the saturated water content, and left to stabilize for 24 h at 20°C.

For both methods of soil amendment, positive controls included KCl, carbendazim, and boric acid for acute toxicity [25], reproduction [23], and avoidance tests [26], respectively (see *Experimental design* for the range of concentrations tested). Chemicals for positive control tests were added in solution along with water to adjust soil moisture to 50 ± 1% of the saturated water content.

*Test organisms*

Earthworms (*E. fetida* and *E. andrei*) were raised in laboratory cultures on moist worm bedding (Carolina Biological Supply) in the dark at 22°C, and fed weekly with a grain-based mixture of carbohydrate, protein, and fat (Magic® Worm Food; Magic Products). Species were not mixed in cultures or in trials. Fully clitellate earthworms weighing between 300 and 700 mg (wet wt) were used in acute toxicity, reproduction, and avoidance tests. Juveniles weighing between 30 and 80 mg (wet wt) were used in the juvenile growth test. Earthworms were acclimatized in moist, untreated soil (artificial or field soil, depending on the test) at 22°C in the dark for at least 24 h prior to each experiment.

*Experimental design*

Test methods were selected to evaluate the effect of exposure to nano-$TiO_2$ on earthworm survival, reproduction, and behavior in artificial and field soils (Table 2). In the survival and reproduction toxicity tests, the experimental treatments

Table 2. Summary of the earthworm tests and soil amendment methods used in the present study

| Test materials[a] | Test | Earthworm species | Soil type | Amendment method | Nominal $TiO_2$ concentrations (mg/kg soil) |
|---|---|---|---|---|---|
| A, E | Reproduction | *Eisenia andrei* | Artificial | Dispersion | 200 |
| A, C, E | Reproduction | *E. andrei* | Artificial, field | Dry mix | 10,000 |
| A, B, C, D, E | Avoidance | *E. andrei* | Artificial | Dry mix | 100–10,000 |
| A, E | Survival, reproduction, juvenile growth | *Eisenia fetida* | Field | Dispersion | 20,200 |
| A, C, E | Reproduction | *E. fetida* | Artificial | Dry mix | 10,000 |

[a] The $TiO_2$ material nominal size and mineral phase: A = 5 nm, anatase; B = 10 nm, anatase; C = 21 nm, 80% anatase, 20% rutile; D = 300 nm, anatase; E = < 44 μm anatase.

(positive control, negative control, material A, and material E) were assigned randomly, and four replicates of each experimental unit (1-L glass jar) were prepared. Material C was also used in the reproduction tests, which included a concentration of 10,000 mg/kg $TiO_2$. Experimental methods were validated with standard concentration–response tests for survival (0, 500, 1,000, 3,500, 5,250, 6,750, 7,500, 8,500, and 9,500 mg KCl per kilogram of soil) or reproduction (0, 0.8, 1.4, 2.5, 3.3, 4.4, and 7.9 mg carbendazim per kilogram soil). Each positive control test used four replicates. No $Ti^{4+}$ ion control was included because the log of activities for the $Ti^{4+}$ free ion and hydroxide species range from $10^{-16}$ to $10^{-21}$ (Visual MINTEQ, Ver 2.53; http://www.lwr.kth.se/English/OurSoftware/vminteq/), so dissolution products from the $TiO_2$ surface were probably insignificant. Soil moisture content (gravimetric determination at 105°C for 24 h) and pH (1:2 soil:water slurry) were determined for each treatment at the start of the test and following adult and juvenile harvests.

The Organization for Economic Co-operation and Development protocols [23,25] were followed for the survival and reproduction toxicity tests. Modifications to protocols are noted in the text. Briefly, 10 earthworms (*E. fetida* or *E. andrei*) were gently rinsed, patted dry with a Kimwipe® tissue, weighed, and added to each prepared 1-L glass jar (as described above under *Soil preparation*). Jars were covered with perforated metal lids and placed in an environmental chamber (Conviron) at 20°C with approximately 65% ambient humidity. Jars were kept in dark conditions to avoid any confounding effects caused by photoexcitation of the $TiO_2$ particles [17]. Soil moisture was assessed by weighing jars and was replenished on a weekly basis. In the reproduction tests, 3 g Magic Worm Food was added to each jar on days 2, 7, 14, 21, and 28. All uneaten food was removed 6 d after each feeding. Earthworms were removed, rinsed, counted, and weighed after 14 d in the survival test and after 28 d in the reproduction test. In the reproduction test, jars were replaced in the environmental chamber and left for a further 28 d, after which unhatched cocoons, hatched cocoons, and juveniles were counted. In the juvenile growth test, 10 juvenile earthworms (*E. fetida*) were gently rinsed, patted dry with a Kimwipe tissue, weighed, and added to each 1-L glass jar. Jars were placed in an environmental chamber, as described previously, and soil moisture was assessed and replenished on a weekly basis. One gram of Magic Worm Food was added weekly for the first six weeks, and thereafter 2 g were added each week. Juveniles were removed, counted, and weighed after 18 weeks.

Avoidance tests were conducted with *E. andrei* exposed to nano-$TiO_2$ amended artificial soil, following the International Organization for Standardization earthworm avoidance test protocol [26]. Alternate sections of six-chambered stainless-steel avoidance rings (Fig. 1) were filled with 300 g moist $TiO_2$-amended or 300 g control soil, which were prepared using the dry-mix method. Each avoidance ring contained no more than one treated soil and one control soil. The alternative soil was either a negative control (with water-only amended soil) or a soil amended with a nominal concentration of micrometer-sized $TiO_2$ equal to that of the nano-$TiO_2$, as described in the *Results* section. Each trial consisted of at least three replicates (rings) plus negative and positive controls. A standard dose–response test consisting of six concentrations of boric acid (0, 200, 360, 630, 1,125, and 2,000 mg boric acid per kilogram soil), each performed in triplicate, was conducted to validate the experimental methods. Soil moisture content and pH were recorded at the start and end of each trial. In total, 10 earthworms

Fig. 1. Six-chambered, stainless-steel earthworm avoidance test unit. Alternate chambers are filled with treatment and control soils. Earthworms move from the central cavity to one of the peripheral chambers and are then free to move between these chambers. External ring diameter measured 240 mm; internal ring diameter measured 60 mm. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

(*E. andrei*) were gently rinsed, patted dry with a Kimwipe tissue, and placed sequentially into the central cavity of each ring. The earthworms moved through one of the six small central openings into a soil-filled chamber, and each subsequent earthworm was added after the previously added earthworm had fully entered one of the chambers. Earthworms could move between the chambers via perforations in chamber walls. The rings were sealed with steel covers and placed in the Conviron environmental cabinet at 20°C with approximately 65% ambient humidity and no light. After 48 h, the steel lid was removed from each ring and the interchamber openings were sealed with steel plates. The number of earthworms in each chamber was recorded and summed to give the total number of earthworms in each treatment group. Avoidance net response is expressed as $NR = ([C - T]/N) \times 100$, where $NR$ is net response, $C$ is the number of worms observed in the control soil, $T$ is the number of worms observed in test soil, and $N$ is the total number of worms per replicate [26]. A 0% avoidance signifies an equal distribution of the 10 earthworms between the three control and three amended-soil chambers, and a 100% avoidance signifies that all 10 earthworms were located in the three control chambers at the end of the experiment. Tests were rejected if the mean earthworm avoidance in the negative control trials was greater than 20%, and replicates were rejected if fewer than nine live earthworms remained in the soil at the end of the experiment.

### *Statistical analysis*

Survival, juvenile growth, and reproduction test responses were analyzed using analysis of variance. Results were considered significantly different at $p \leq 0.05$. In the avoidance test, the distribution of earthworms between the amended and control soils at the end of each trial was analyzed using the two-tailed binomial distribution test with an expected normal distribution of earthworms between the two soil types (expected $p = 0.5$) [27]. If significantly fewer earthworms were found in the amended soil than in the control soil at the end of the test compared with the expected binomial distribution ($p \leq 0.05$), but the mean avoidance was <60%, the response was defined as weak avoidance. If significantly fewer earthworms were found in the amended soil than in the control soil at the end of the test, and the mean avoidance was ≥60% (i.e., eight of the 10 earthworms in each avoidance ring found in the control soil after 48 h), the response was deemed strong avoidance. The latter follows the International Organization for Standardization protocol, in which soils eliciting an avoidance response of at least 60% are defined as having a limited habitat function [26]. Data were expressed as the mean ± SD. The median lethal concentration (LC50) and median effective concentration (EC50) values were calculated in ToxCalc® version 5.0.18 (Tidepool Scientific Software) and Excel Analytical ToolPak (Microsoft Office Excel 2007), respectively. All other statistical analyses were performed in SAS® for Windows, Version 9.2 (SAS Institute).

## RESULTS

### *Material characterization*

X-ray diffraction revealed that all $TiO_2$ materials were composed of 100% anatase, except for material C, which was composed of 83% anatase and 17% rutile, similar to the manufacturers' specifications (Table 1). Transmission electron microscopic images revealed considerable differences in particle sizes and morphologies between the nano-$TiO_2$ test materials (Fig. 2). All nanomaterials were agglomerated. Material A appeared to have two forms. In the first form, primary particles were discernible in the agglomerates (Fig. 2a), but, in the second form, particles appeared to be more intimately aggregated, with no individual particles observed (Fig. 2b). The terms "agglomerate" (a group of particles held together by relatively weak forces [28]), and "aggregate" (a heterogeneous particle in which the various components are not easily broken apart [28]), will be used in the present article to differentiate between the two forms. The primary particle size in material A (mean measured diameter of 22 ± 7 nm) was approximately four times greater than the manufacturer-reported size (5 nm nominal size). No primary particles were identified in material B, which appeared to be composed of rough-surfaced aggregates similar to those observed in material A (Fig. 2c). Primary particle size measurement was not possible for material B. The morphology of material B was consistent with reports from other research groups who used similar batches of this nanomaterial [8]. Agglomerated primary particles from material C (mean measured diameter of 19 ± 4 nm) were clearly visible (Fig. 2d). Primary particles were also discernible in material D and E agglomerates and included both micrometer- and nanometer-sized primary particles (mean measured diameters 119 ± 46 nm and 118 ± 38 nm, respectively, Fig. 2e and f).

The BET-measured SSA of material B (274 $m^2$/g) was nearly twice that of material A and five times greater than that of material C (Table 1). The point of zero charge measurements for the three nano-$TiO_2$ test materials were between pH 6.20 and pH 6.32 (Table 1), within the range reported by other researchers [29]. Micrometer-sized materials had lower than expected point of zero charge values (below pH 3; Table 1), which were consistent in tests run on three separate occasions. The reason for the low point of zero charge values for the micrometer-sized $TiO_2$ is not known. Dynamic light scattering measurements in dispersions with pH 6.60 to 6.80 revealed considerable agglomeration in all nano-$TiO_2$ dispersions (Table 1). Agglomerate average sizes ranged from 805 nm for material B to 1,209 nm for material C. Agglomerate size was smaller in the micrometer-sized materials, ranging from 258 to 298 nm.

Nano-$TiO_2$ particles tend to agglomerate rapidly in aqueous solutions, but agglomerate size decreases as the pH moves away from the point of zero charge [30]. The mean agglomerate size of material A decreased from 900 nm at pH 6.7 to 590 nm at pH 10.0, and the smallest agglomerate size detected by dynamic light scattering was reduced from 534 nm (pH 6.7) to 120 nm diameter (pH 10.0; Table 1). Therefore, soils amended with the dispersion method ($TiO_2$ materials in solution at pH 10) should contain small nano-$TiO_2$ agglomerates without further

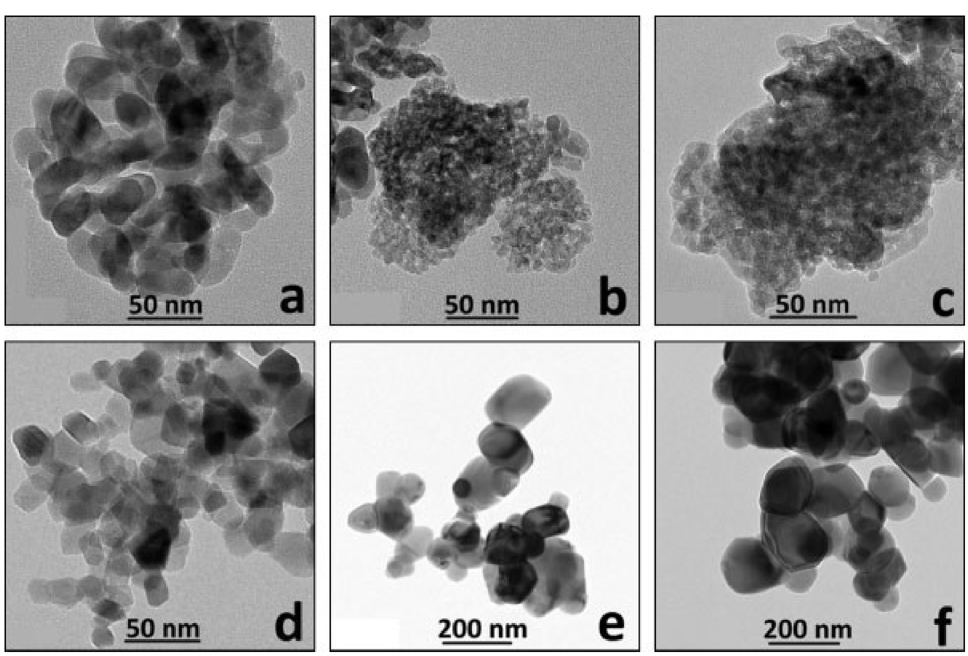


Fig. 2. Transmission electron microscopic images of the titanium dioxide samples used in the present study: material A, form with particles (**a**), material A, form with no particles discernible (**b**), material B (**c**), material C (**d**), material D (**e**), and material E (**f**).

sonication or use of chemical dispersants. However, the $TiO_2$ solutions (60 and 600 mg/L) for the dispersion method could not be measured by dynamic light scattering, so the size of agglomerates added to the soil with this method is not known. Soil pH returned to original values within hours of adding the dispersion solutions (pH 10) because of the soils' ability to buffer changes in hydrogen ion activity (data not shown). There was no significant difference in soil pH or moisture content between the treatment and control soils at the start or end of any test described in the next section.

Commercial $TiO_2$ particles were virtually free of trace elements (concentrations below 2 μg/g; data not shown), except for material A (17 μg/g Pb) and material B (5 μg/g Ni); however, all contained Al at concentrations ranging from 2 μg/g (material C) to 40 μg/g (material B). The X-ray fluorescence analysis of experimental soils revealed that the unamended agricultural and artificial soils contained the equivalent of 0.45% and 0.11% $TiO_2$, respectively (data not shown).

*Earthworm tests*

Exposure to nano-$TiO_2$-amended soil had no significant effect on earthworm survival up to 200 mg $TiO_2$ per kilogram of soil in standard survival tests (100% survival in all treatments, data not shown). Earthworm survival was also 100% in all reproduction tests using soil amended with up to 10,000 mg/kg nano-$TiO_2$ (data not shown). Standard survival tests were not conducted at 10,000 mg/kg nano-$TiO_2$ because no mortality was observed in range-finding survival and reproduction tests at this concentration. Earthworms harvested from soils amended with nano-$TiO_2$ displayed no obvious signs of ill health in the survival and reproduction tests. Survival and growth of juvenile earthworms exposed to nano-$TiO_2$-amended soil (material A) for up to 18 weeks were similar to those for juveniles exposed to soil amended with micrometer-sized material (material E) and in unamended control soils (Table 3). There was no significant effect ($p > 0.05$) on the number of cocoons produced, hatching rate, or number of juveniles produced by adult *E. fetida* or *E. andrei* earthworms in either field or artificial soil with exposures up to 10,000 mg $TiO_2$ per kilogram of soil compared with negative or micrometer-sized controls (Table 3). The reproductive rate of *E. andrei* was up to twice as high as that of *E. fetida*, depending on the experimental conditions. The higher reproductive rate for *E. andrei* concurs with previously reported results [31]. Positive control test results complied with laboratory control charts (survival test using KCl, $LC50 = 5{,}975$ mg KCl per kilogram soil, reproduction test cocoon production using carbendazim, $EC50 = 3.3$ mg carbendazin per kilogram soil, data not shown).

Despite the lack of effect of nano-$TiO_2$ on adult survival and reproduction (Table 4) and the earthworms' apparent good health (no visible sign of ill effects) at the end of the reproduction tests, there were significantly fewer earthworms in soils amended with all three types of nano-$TiO_2$ than in the negative control soils after 48 h (Table 5) in the avoidance tests (conducted in artificial soil only). Four separate avoidance tests using 10,000 mg material A per kilogram of soil were conducted over a span of four months (about 2, 12, and 16 weeks after the first test, each using freshly amended soil as described in *Materials and Methods*). In all tests, significantly fewer earthworms were found in the amended soil than in the control soil at the end of the test ($p \leq 0.05$; Table 5), and in three of the tests a strong avoidance response (≥60% avoidance, as described in *Materials and Methods*) was observed. A weak avoidance response (also described in *Materials and Methods*) was observed in soils amended with 1,000 mg/kg of materials A and C or 5,000 mg/kg of material B ($p \leq 0.05$; Table 5). At other concentrations tested (Table 5), no significant avoidance of nanomaterial-amended soil was observed. Distribution of earthworms in soils amended with up to 10,000 mg/kg of the micrometer-sized materials D and E was not significantly different from the expected binomial distribution.

In a separate study, earthworms were given the choice between soils amended with either 10,000 mg/kg of material E or a similar concentration of one of the three nanomaterials

Table 3. Number of cocoons and juveniles produced by *Eisenia* spp. in soils amended with nanometer- and micrometer-sized titanium dioxide ($TiO_2$)

| Test materials[a] | Nominal concn. $TiO_2$ (mg/kg soil)[b] | Species | Soil[c,d] | Total cocoons (no.)[d,e] | Juveniles hatched (no.)[d,e] | Hatch rate (%)[d,f] |
|---|---|---|---|---|---|---|
| A | 200 | *Eisenia andrei* | Artificial (i) | 62 (5) | 115 (13) | 76 (8) |
| E | 200 | | | 63 (8) | 160 (19) | 90 (5) |
| Negative control | 0 | | | 51 (9) | 121 (44) | 79 (7) |
| A | 10,000 | *E. andrei* | Field (ii) | 96 (4) | 220 (8) | 94 (6) |
| C | 10,000 | | | 96 (9) | 224 (14) | 90 (4) |
| E | 10,000 | | | 88 (7) | 233 (35) | 93 (3) |
| Negative control | 0 | | | 97 (8) | 252 (47) | 87 (9) |
| A | 10,000 | *E. andrei* | Artificial (ii) | 94 (3) | 208 (34) | 90 (2) |
| C | 10,000 | | | 102 (8) | 219 (77) | 88 (3) |
| E | 10,000 | | | 102 (11) | 232 (39) | 90 (2) |
| Negative control | 0 | | | 90 (18) | 197 (81) | 91 (7) |
| A | 20 | *E. fetida* | Field (i) | 66 (5) | 64 (47) | 44 (26) |
| A | 200 | | | 70 (10) | 99 (77) | 55 (40) |
| E | 20 | | | 64 (16) | 110 (88) | 61 (32) |
| E | 200 | | | 73 (11) | 79 (69) | 54 (35) |
| Negative control | 0 | | | 70 (17) | 78 (75) | 34 (8) |
| A | 10,000 | *E. fetida* | Artificial (ii) | 51 (10) | 62 (9) | 85 (5) |
| C | 10,000 | | | 42 (11) | 67 (18) | 84 (6) |
| E | 10,000 | | | 46 (15) | 64 (16) | 81 (10) |
| Negative control | 0 | | | 49 (9) | 55 (23) | 84 (9) |

[a] Materials A and C were nanometer-sized $TiO_2$ and material E was micrometer-sized $TiO_2$.
[b] The negative control soil in the dispersion method had the same pH and $Na^+$ concentration as the $TiO_2$-amended soils.
[c] (i) and (ii) indicate dispersion and dry-mix method of amendment addition, respectively.
[d] Numbers in parentheses are standard deviations ($n = 4$). Treatment results are not significantly different from control results, unless indicated.
[e] Adults were removed after 28 d, and the numbers of cocoons and juveniles were recorded after 56 d.
[f] The hatch rate is defined as (number of hatched cocoons/total number of cocoons) × 100.

Table 4. Survival and growth in *Eisenia fetida* juveniles raised in field soils amended with nanometer- and micrometer-sized titanium dioxide ($TiO_2$)

| Material[a] | Nominal concn. $TiO_2$ (mg/kg soil)[b] | Survival (%)[c] | Increase in body weight (%)[c,d] |
|---|---|---|---|
| A | 20 | 100 (0) | 504 (130) |
| A | 200 | 100 (0) | 582 (68) |
| E | 20 | 100 (0) | 540 (141) |
| E | 200 | 100 (0) | 513 (156) |
| Negative control | 0 | 100 (0) | 602 (164) |

[a] Material A was nanometer-sized $TiO_2$ and material E was micrometer-sized $TiO_2$. The negative control soil had the same pH and $Na^+$ concentration as the $TiO_2$-amended soils.
[b] Soils were amended using the dispersion method.
[c] Numbers in parentheses are standard deviations ($n = 4$). Treatment results are not significantly different from control results, unless indicated.
[d] The increase in body weight is defined as (final total earthworm wet weight/original total earthworm wet weight) × 100. Final weight was measured after 84-d exposure to amended test soils.

(material A, material B, or material C). Earthworms displayed weak avoidance of soils amended with material A, preferring the soils amended with material E ($p \leq 0.05$; Table 6). However, no significant avoidance of soils amended with materials B or C was observed.

A positive relationship was observed between the percentage earthworm avoidance and $TiO_2$ SSA at an amendment concentration of 10,000 mg $TiO_2$ per kilogram soil for all materials except for material B (Fig. 3), with the mean avoidance increasing from −7% to 73% as the $TiO_2$ SSA increased from 8 $m^2$/g to 141 $m^2$/g (Table 1). Earthworms were normally distributed in the negative control, and results of positive control tests using boric acid were within the limits set in this laboratory (avoidance test EC50 = 3,600 mg boric acid per kilogram soil).

Table 5. *Eisenia andrei* avoidance of artificial soils amended with nanometer- and micrometer-sized titanium dioxide ($TiO_2$) using unamended soil as an alternative soil

| Test material[a] | Test number[b] | Nominal concentration $TiO_2$ (mg/kg soil) | Avoidance (%)[c,d] | $n$[e] |
|---|---|---|---|---|
| A | Test 1 | 100 | −27 (23) | 3 |
| | | 1,000 | 33 (12)[f] | 3 |
| | | 10,000 | 80 (20)[f,g] | 3 |
| A | Test 2 | 100 | 0 (20) | 3 |
| | | 1,000 | 13 (23) | 3 |
| | | 5,000 | 1 (41) | 5 |
| | | 10,000 | 93 (12)[f,g] | 3 |
| A | Test 3 | 10,000 | 80 (14)[f,g] | 5 |
| A | Test 4 | 10,000 | 48 (23)[f] | 5 |
| B | Test 4 | 1,000 | −13 (31) | 3 |
| | | 5,000 | 40 (20)[f] | 3 |
| | | 10,000 | 24 (33) | 5 |
| C | Test 4 | 100 | −14 (49) | 4 |
| | | 1,000 | 45 (44)[f] | 4 |
| | | 10,000 | 37 (15)[f] | 6 |
| D | Test 4 | 10,000 | 33 (42) | 3 |
| E | Test 4 | 100 | −20 (81) | 5 |
| | | 1,000 | 4 (17) | 5 |
| | | 10,000 | −7 (37) | 6 |
| Negative control | Test 4 | 0 | −8 (49) | 8 |

[a] Materials A, B, and C were nanometer-sized $TiO_2$; Materials D and E were micrometer-sized $TiO_2$.
[b] Number of the repeat tests using Material A. Subsequent tests were conducted 2, 12, and 16 weeks after the initial test.
[c] Zero percent avoidance indicates equal distribution of earthworms between the two soils, and 100% avoidance indicates that all earthworms were found in the control soil at the end of the test. Numbers in parentheses are standard deviations.
[d] The negative control results (unamended soils) for all tests are summed and displayed as a zero concentration of $TiO_2$.
[e] $n$ is the number of replicates (avoidance rings). A total of 10 earthworms were placed in each ring.
[f] $p \leq 0.05$ (binomial distribution test) based on the expected equal distribution of earthworms between treatment and control soils at harvest.
[g] Significant avoidance (avoidance ≥ 60%).

## DISCUSSION

Earthworm survival and reproduction test results indicate that exposure to as much as 10,000 mg $TiO_2$ per kilogram soil does not significantly affect the number of *Eisenia* spp. adults or their offspring. In contrast, Heckmann et al. [10] reported a 49% reduction in the number of juveniles produced by *E. fetida* following 28 d of exposure to 1,000 mg $TiO_2$ per kilogram soil using a material with characteristics similar to material C in the present study. It is not clear why *Eisenia* spp. exhibited different responses to nano-$TiO_2$ in these studies. The disparity in results between apparently similar tests could arise from differences in test organisms, soils, or experimental methods and emphasizes the current lack in understanding of mechanisms controlling interactions between nanoparticles, organisms, and natural media [8,18,32].

The nanomaterial characteristics reported in the present study (Table 1) relate to the starting nanomaterials, and, although these properties may assist in reproducing these tests and allow for future meta-analysis of the data, they do not necessarily reflect the form and interactions of the nanomaterials once they have been added to the soils [18]. When nano-$TiO_2$ is added to simple aqueous solutions containing dissolved organic matter, phosphate, or calcium, these constituents rapidly coat the nanomaterial surfaces [30,33], and similar processes likely occur in complex media such as soils. Furthermore, the chemical and physical properties of natural soils likely control the aggregation, agglomeration, and reactivity of nanomaterials, but how such reactions change in soils with different physicochemical properties is not yet understood [32]. Background Ti concentrations in agricultural and artificial soils in the present study were equivalent to 2,700 and 660 mg Ti per kilogram of soil, respectively. Given the nominal concentration of nano-$TiO_2$ and micrometer-$TiO_2$ added to soils in the current tests (200–10,000 mg/kg), the increase in measurable Ti ranged between 0.04% and 900%. Given the high values of natural Ti and the abundance of natural nanometer-sized objects in soils [34], tracking transformations of engineered $TiO_2$ particles in soils presents considerable challenges. Further studies on the fate of engineered $TiO_2$ in soil, perhaps using radioisotopes as tracers [35], would provide insight into its interactions with soil constituents and the forms in which it is encountered by soil organisms and help to clarify the factors that might lead to different results from seemingly similar tests.

Earthworms avoided soils amended with concentrations of 1,000 mg nano-$TiO_2$ per kilogram soil or higher (Table 5), but no response was observed in soils amended with micrometer-sized $TiO_2$, and in one test the earthworms preferred soil amended with micrometer-sized $TiO_2$ to soil amended with nanometer-sized $TiO_2$ (Table 6). Nanometer-sized $TiO_2$ is composed of smaller primary particles than its micrometer-sized counterpart, and this leads to the hypothesis that avoidance behavior is related to differences in fundamental material properties, such as SSA or primary particle size. Dissolution products from $TiO_2$ are unlikely to play a role in toxicity because of its extremely low solubility. There was also no significant difference in soil pH and moisture content between

Table 6. *Eisenia andrei* avoidance of artificial soils amended with 10,000 mg/kg nanometer-sized titanium dioxide ($TiO_2$) using soil amended with 10,000 mg/kg micrometer-sized $TiO_2$ as an alternative soil

| Test material[a] | Nominal concentration $TiO_2$ (mg/kg soil) | Avoidance (%)[b] | $n$[c] |
|---|---|---|---|
| A | 10,000 | 58 (29)[d] | 3 |
| B | 10,000 | 16 (22) | 5 |
| C | 10,000 | 27 (31) | 3 |

[a] Materials A, B, and C were nanometer-sized $TiO_2$; material E was micrometer-sized $TiO_2$.
[b] Zero percent avoidance indicates equal distribution of earthworms between the two soils, and 100% avoidance indicates that all earthworms were found in the control soil at the end of the test. Numbers in parentheses are standard deviations.
[c] $n$ = number of replicates (avoidance rings). Ten earthworms were placed in each ring.
[d] $p \leq 0.05$ (binomial distribution test) based on the expected equal distribution of earthworms between treatment and control soils at harvest.

treatments, and trace element concentrations in the nanomaterials were considerably lower than those reported to cause earthworm avoidance [36].

A positive relationship was observed between earthworm avoidance and the SSA of the $TiO_2$ materials in soils amended with 10,000 mg/kg $TiO_2$ (Fig. 3), although this could not be quantified because of the high variability in response, especially at the lower SSA values. The response to material B was weaker than anticipated, based on its comparatively high BET-measured SSA (274 $m^2$/g; Table 1). The high SSA of material B may be attributed in part to the presence of nanometer-sized mesopores [37], but it is not known whether these internal surfaces affect processes leading to the avoidance behavior of earthworms. Although $TiO_2$ SSA appears to be positively related to reactions causing the earthworm avoidance response, more testing with well-characterized mesoporous $TiO_2$ nanomaterials is required to evaluate the contribution of internal nanomaterial surfaces to avoidance. Submicrometer-sized aggregates in materials A and B, as well as agglomeration of all $TiO_2$ materials in solution, made it difficult to define the primary particle size (Fig. 2). Therefore, particle size was not a meaningful exposure metric in the present study, and no relationship was elucidated between $TiO_2$ primary particle size and earthworm avoidance behavior.

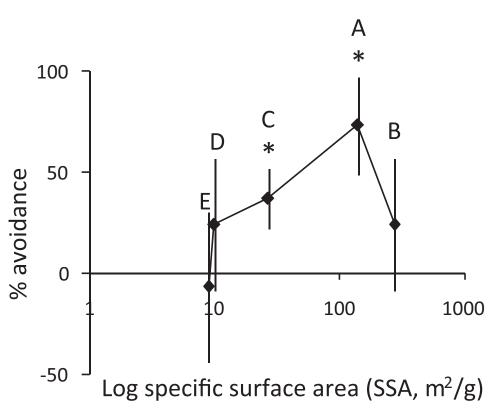


Fig. 3. Relationship between earthworm avoidance behavior at 10,000 mg/kg titanium dioxide ($TiO_2$) and the Brunauer, Emmett, and Teller measured specific surface area (SSA) of nano-$TiO_2$ materials (A–C) and micrometer-sized $TiO_2$ materials (D,E). See Table 1 for physical characterization of the $TiO_2$ test samples. The percentage avoidance is described in Table 5. $^*p \leq 0.05$, based on the expected equal distribution of earthworms between treatment and control soils at harvest (binomial distribution test). Error bars marked above and below the points indicate standard deviations, where $n$ ranged from 3 to 5 depending on the experiment.

The reasons earthworms avoid soils amended with nano-$TiO_2$ are not clear, but results of previous studies on the behavior of earthworms and reactions of $TiO_2$ describe effects that might be implicated in this response and merit further investigation. Earthworms have demonstrated sensitivity to soils containing trace metals such as Zn, Pb, and Cu and will avoid soils contaminated with these metals at concentrations below those affecting survival or reproduction but that may induce sublethal stress [12,14]. In the present study, earthworms avoided soils amended with nano-$TiO_2$ when they had the opportunity to move into clean soil (Table 5), despite no observable negative effect on their health. A recent study reported DNA damage and increased activities of enzymes associated with oxidative stress in *E. fetida* following exposure to nano-$TiO_2$ in soil at concentrations similar to those to which earthworms were exposed in the present study and concluded that nano-$TiO_2$ does indeed exert a negative effect on earthworm physiology, possibly through production of reactive oxygen species [9]. Whether the avoidance observed in the present study was a direct response to nano-$TiO_2$ or its reaction products, such as reactive oxygen species, requires further investigation. Gut and soil bacteria play an important role in earthworm digestion [38]. Bacterial numbers in aquatic systems were reduced following exposure to nano-$TiO_2$ even in the absence of light [39,40]; these findings may imply that nano-$TiO_2$ exerts an indirect effect on earthworm behavior through its inhibitory effect on microorganisms.

The influence of the adsorptive properties of $TiO_2$ surfaces on the soil environment should also be considered. Laboratory experiments demonstrated that nano-$TiO_2$ readily adsorbs both ions and humic materials commonly found in the soil solution [30,33], and naturally occurring $TiO_2$ in soils is thought to play a role in the retention and availability of nutrient ions through surface adsorption of dissolved inorganic and organic molecules [41]. The engineered $TiO_2$ used in the present study was uncoated and freshly added to the soil, and the three nanomaterials had considerably higher SSAs than the micrometer-sized materials ($\geq$49 $m^2$/g compared with $\leq$13 $m^2$/g; Table 1). It is possible that changes in soil solution chemistry in nanomaterial-amended soils, caused by the addition of strongly adsorptive nanoparticles, rendered the soil less palatable to the earthworms. Testing of this hypotheses will increase our understanding of the mechanisms underlying the observed earthworm avoidance response to nano-$TiO_2$-amended soils.

Earthworm tests were conducted with two closely related earthworm species in one natural soil and one artificial soil freshly amended with uncoated $TiO_2$ materials, so care should be taken when extrapolating the results from the present study to natural environments for ecological risk assessment purposes. A recent article estimated the upper quantile value for the increase of nano-$TiO_2$ on American sludge-treated soils to be 179 μg $TiO_2$ per kilogram soil per annum [4]. The present study found that exposure for at least 28 d to soil amended with up to 10,000 mg/kg nano-$TiO_2$ had no significant effect on earthworm survival, juvenile growth, or reproductive parameters (Tables 3 and 4) and that earthworms did not avoid soils amended with up to 100 mg/kg nano-$TiO_2$ (Table 5). These results suggest that the levels of the nano-$TiO_2$ likely to be found in sludge-treated soils will not present a significant risk to earthworm populations. That more than 100 mg of freshly applied nano-$TiO_2$ per kilogram soil was required to elicit an earthworm avoidance response, compared with the significant avoidance response observed to soil amended with 9 mg/kg Ag nanomaterials [15], is further indication of the comparatively low toxicity of nano-$TiO_2$. A logical next step will be to study the behavior of representative earthworms species exposed to a range of well-characterized nano-$TiO_2$ materials that were previously aged in natural soils or sludges at environmentally relevant concentrations. More research is needed to evaluate the effects of earthworm exposure to nano-$TiO_2$ under environmental conditions that could be experienced by terrestrial organisms in the coming decades.

## CONCLUSIONS

Nanometer-sized $TiO_2$ had no significant effect on earthworm survival, juvenile growth, or reproductive parameters when mixed in artificial or field soil. However, earthworms were able to detect and avoid artificial soils amended with nano-$TiO_2$, although the reasons for these responses are not clear at present. The findings reported herein indicate that an earthworm behavioral test was more sensitive to the effects of nanomaterials (using nano-$TiO_2$ as an example) than standard lethality and reproduction toxicity assays. Although the earthworm avoidance response appeared to be related to the SSA of nano-$TiO_2$, challenges remain in characterizing material properties in natural media, which are essential for interpreting results from nanoecotoxicological studies. It is proposed that earthworm sensitivity to nano-$TiO_2$ may be a direct response to the stimulation of receptor cells or an indirect response to changes in the soil solution chemistry or a result of the effect of nano-$TiO_2$ on microorganisms. More research is required to investigate these potential mechanisms and to determine whether earthworms also avoid soils and sludges containing nano-$TiO_2$ that have been modified through natural processes (aged). The high concentrations of the nano-$TiO_2$ materials required to elicit the earthworm avoidance response in the present study indicate that predicted levels of nano-$TiO_2$ in sludge-treated agricultural soils are unlikely to pose a significant risk to earthworm populations.

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