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Earthworm Sublethal Responses to Titanium Dioxide Nanomaterial in Soil Detected by ^{1}H NMR Metabolomics

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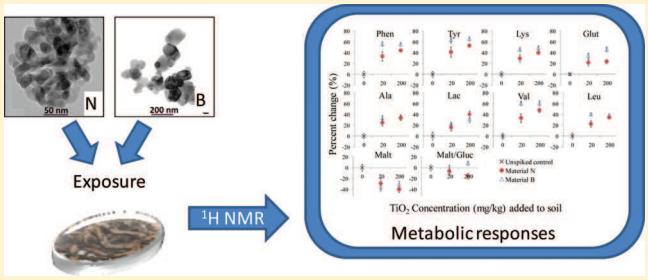
Supporting Information

ABSTRACT: ^{1}H NMR-based metabolomics was used to examine the response of *Eisenia fetida* earthworms raised from juveniles for 20−23 weeks in soil spiked with either 20 or 200 mg/kg of a commercially available uncoated titanium dioxide (TiO_2) nanomaterial (nominal diameter of 5 nm). To distinguish responses specific to particle size, soil treatments spiked with a micrometer-sized TiO_2 material (nominal diameter, <45 μm) at the same concentrations (20 and 200 mg/kg) were also included in addition to an unspiked control soil. Multivariate statistical analysis of the ^{1}H NMR spectra for aqueous extracts of *E. fetida* tissue suggested that earthworms exhibited significant changes in their metabolic profile following TiO_2 exposure for both particle sizes. The observed earthworm metabolic changes appeared to be consistent with oxidative stress, a proposed mechanism of toxicity for nanosized TiO_2. In contrast, a prior study had observed no impairment of *E. fetida* survival, reproduction, or growth following exposure to the same TiO_2 spiked soils. This suggests that ^{1}H NMR-based metabolomics provides a more sensitive measure of earthworm response to TiO_2 materials in soil and that further targeted assays to detect specific cellular or molecular level damage to earthworms caused by chronic exposure to TiO_2 are warranted.



INTRODUCTION

Traditional pigment grade titanium dioxide (TiO_2; primary particle size range 200−300 nm) is widely manufactured for use as an opaque white pigment in paints, plastics, foods, pharmaceuticals, cosmetics, and other products.[1] TiO_2 is also a common ingredient in sunscreens due to its efficient absorption of UV light.[1] Since opacity is not required for this application, manufacturers are increasingly utilizing a much smaller particle size (10−20 nm) of TiO_2, which provides a transparent appearance.[1,2] These smaller particles meet the definition of manufactured nanoparticles, described as any synthetic material with one or more dimension less than 100 nm.[3] Although TiO_2 particles with diameter >100 nm are considered toxicologically inert,[1] it has been suggested that the greater relative surface area of nanosized particles may cause them to be more biologically active and increase their toxicity.[4] TiO_2 nanomaterials that enter the environment during their production, use, or disposal may be deposited in soil or accumulate in sewage treatment plant sludge that may subsequently be applied to soil.[5,6] Preliminary evidence suggests that, once present in the soil, these nanomaterials may bioaccumulate in terrestrial organisms and thereby enter the terrestrial food web.[7−10] A better understanding of the toxicity of TiO_2 nanomaterials to terrestrial organisms such as earthworms is needed to determine their long-term impact in terrestrial environments.

Some recent studies that have examined the toxicity of nanosized TiO_2 to earthworms noted that earthworms expressed toxic responses to nanosized TiO_2 only at very high concentrations in soil (≥1000 mg/kg).[11,12] These concentrations exceed predicted maximum TiO_2 concentrations of 0.3 mg/kg in soil and up to 523 mg/kg in sewage treatment plant sludge,[5,6] so it has been suggested that TiO_2 nanomaterials may be nontoxic to earthworms at environmentally relevant concentrations. However, Lapied et al.[13] noted increased apoptotic frequency in the cuticle and intestinal epithelium of *Lumbricus terrestris* earthworms after seven days of exposure to 100 mg/L nanosized TiO_2 in water, and a similar trend was noted in earthworms exposed to only 15 mg/kg nanosized TiO_2 in soil after four weeks of exposure. Therefore, the toxicity of TiO_2 nanomaterials to earthworms

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Table 1. Selected Characteristics of Titanium Dioxide Materials Used in This Study

material	mineral phase	nominal particle size[a] (nm)	TEM imaged particle size[b] (nm)	specific surface area[c] (m^2/g)	mean agglomerate hydrated diameter[d] (nm)	minimum agglomerate hydrated diameter[d] (nm)	point of zero charge[e] (pH)
N	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	<45 000	118 (±38)	9	298	181	<3.50

[a]Please refer to Supporting Information or McShane et al.[27] for complete description of methods used to determine these values. Nominal size is the particle diameter reported by the manufacturer. [b]Transmission electron microscopy (TEM)-imaged size was estimated by counting 40−50 particles in 4−6 TEM images. Numbers in brackets are standard deviations. Samples were sonicated in methanol for 1 s prior to imaging. [c]Specific surface area was measured on the delivered powders. [d]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 6.6−6.8 unless specified. [e]Measured using dynamic light scattering. Samples (dispersion concentrations described above) were vortexed for 3 min before measurement.

following chronic exposure to low, environmentally relevant concentrations remains uncertain.

To further investigate the response of earthworms to chronic sublethal exposures to TiO_2 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 TiO_2. To distinguish responses specific to particle size, this experiment also included soil treatments spiked with TiO_2 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 TiO_2-treated and control soil exposures was performed using ^{1}H NMR 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.[14] 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[15−22] and has also been applied to identify various mechanisms of toxicity of nanosized TiO_2 in rats.[23] 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.[24] Therefore, metabolomics is well suited for the preliminary assessment of the biological response of earthworms to nanosized TiO_2, since it provides a nonspecific assessment of the end result of multiple simultaneous biological processes.[20,23−25]

EXPERIMENTAL SECTION

Soil Spiking with TiO_2. All chemicals were analytical grade, and ultrapure water (Millipore-type, 18 MΩ/cm) was used unless otherwise indicated. Soil for this study was collected from a field at the MacDonald Campus farm (Ste-Anne-de-Bellevue, Quebec, Canada; 45°30′ N, 73°35′ W). This soil is a Typic Hapludalf sandy loam of the Chicot series, with a CEC of 12.2 cmol charge/g ($BaCl_2$ extraction[26]), pH of 6.7, 5% soil organic matter (loss on ignition at 360 °C), and 50% water (w/w) at field capacity. The soil clays in this area are dominated by Illite. This soil contained 0.27% endogenous Ti (X-ray fluorescence, fused bead preparation, Philips PW2440 4 kW XRF spectrometer, and rhodium tube, Panalytical, MA). Soil was sieved to 2 mm and stored at 20 °C before use.

The nanosized TiO_2 material (material N, 100% anatase, NanoAmor) used in this study had a nominal particle size of 5 nm and mean transmission electron microscopy (TEM) imaged size of 20 nm (Table 1), and the larger particle sized TiO_2 material (material B, 100% anatase, Sigma-Aldrich) had a nominal particle size <45 μm and mean TEM imaged size of 118 nm (Table 1). Additional characteristics of both materials are provided in Table 1 and the Supporting Information (SI). The methods used for characterization of the materials are described in the SI and in McShane et al.[27] Uncoated TiO_2 materials were selected to minimize complications due to differences in surface treatments.

The five soil treatments prepared included an unspiked control, soils spiked with either 20 or 200 mg/kg of material N (N20 and N200), and soils spiked with either 20 or 200 mg/kg of material B (B20 and B200). The TiO_2 spiked soil treatments were created using a liquid dispersion method. Initially, 250 mg of appropriate TiO_2 material was added to 250 mL water in a polypropylene container. The mixtures were vortexed for 1 min, raised to pH 10 with 0.05 N NaOH (<5 mL added) to decrease particle agglomerate size,[28] vortexed again for 3 min, and left to stabilize for 4 h. The adjustment to pH 10 required less NaOH for material B spiked soils than for material N spiked soil; therefore, sodium chloride solution (0.05 M) was added to the material B dispersion after stabilization to achieve equal amounts of sodium in both dispersions. The total concentration of Na^+ added (0.084 mM Na^+ per kg soil) was below the level shown to impair reproduction and survival in *Eisenia* species.[29] These mixtures were vortexed for 30 s, and subsamples were added to water (in polypropylene containers) to yield mixtures with concentrations of 60 or 600 mg/L TiO_2 which were mixed with sufficient water to bring the soil to 50% of field capacity, vortexed for 30 s, and then added to 100 g air-dry soil in a 500 mL glass treatment jar. Final soil concentrations were 20 or 200 mg TiO_2 per kg soil. An unspiked control (water only) treatment was prepared in a similar fashion, adjusting the pH to 10 with 0.05 N NaOH and adding a total of 0.084 mM Na^+ per kg soil. Finally, all soils were stabilized for 24 h at 20 °C. For all soils, soil pH returned to original values within hours of adding the dispersion solutions because of the soils' ability to buffer changes in hydrogen ion activity (mean pH of 7.0 ± 0.03 standard error, n = 5).

Earthworm Exposure and Extraction for ^{1}H NMR. *E. fetida* earthworms for this study were cultured on moist earthworm bedding (Carolina Biological Supply Company, Burlington, NC) in dark conditions at 22 °C and fed weekly on a grain-based mixture of carbohydrate, protein, and fat (Magic Worm Food, Magic Products Inc., Amherst Junction, WI). Juvenile earthworms were selected for this experiment as they may be more sensitive to environmental contaminants than adult earthworms.[30] To initiate the exposure, 10 juvenile earthworms weighing between 30 and 80 mg (wet weight) were added to each of the five treatment jars. The density of earthworms in the current study (10 earthworms per 100 g dry

soil mass) is higher than that traditionally recommended for toxicity tests using adult earthworms (e.g., 10 earthworms per 500−600 g dry soil mass in OECD methods[31]). This increased density may have caused some additional stress to earthworms as earthworms matured from juveniles to adults. However, as density was consistent between control and exposed treatments, differences in the metabolomic response between earthworms in this study are expected to relate to TiO_2 exposure. Jars were covered with perforated metal lids, wrapped in aluminum foil, and placed in an environmental chamber (Conviron, MB, Canada) at 20 °C and approximately 65% ambient humidity, with no light. Soil moisture was replenished on a weekly basis. Food (1 g) was added weekly for the first six weeks, and thereafter, 2 g of food was added. Uneaten food was removed after seven days. Earthworms were harvested for analysis when they had achieved a wet mass within a target range of 0.3−0.5 g (20−23 weeks of exposure). Earthworm final average wet mass was 0.42 g ± 0.007 g (standard error, n = 50). Throughout the exposure period, we did not observe earthworms on the surface of the soil or any other signs of organism distress. After removal, earthworms were rinsed with water, depurated for 96 h on moist filter paper in individual Petri dishes, then weighed, flash-frozen with liquid nitrogen, lyophilized, reweighed, and stored frozen until extraction.

Lyophilized earthworms were homogenized in a 1.5 mL centrifuge tube using a 5 mm wide stainless steel spatula. The homogenized tissue was extracted using 1.2 mL of a 0.2 M monobasic sodium phosphate buffer solution ($NaH_2PO_4{\cdot}2H_2O$, 99.3%, Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity, Sigma Aldrich) as a biocide and 10 mg/L of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS, 97%, Sigma Aldrich) as an internal standard.[32,33] Buffer solution was made with D_2O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D_2O, Cambridge Isotope Laboratories Inc.). This aqueous buffer was previously demonstrated to extract the largest quantity of the widest variety of metabolites from *E. fetida* tissue samples,[33] and similar aqueous extractions have been applied in several other earthworm metabolomic studies.[17−19,21,34] Samples were vortexed for 30 s using a VX 100 vortexer (Labnet, NJ) and sonicated for 15 min using a FS60 mechanical ultrasonic cleaner (Fisher Scientific). Samples were centrifuged at 14 000 rpm (~15 000 *g*) for 20 min using an International Equipment Company 21000 centrifuge (Fisher Scientific), and the supernatant was transferred into a new 1.5 mL centrifuge tube. Centrifugation was repeated twice, and the final extracts were transferred into 5 mm high throughput-plus NMR tubes (Norell Inc., NJ).

^{1}H NMR Spectroscopy. ^{1}H NMR spectra of the earthworm extracts were acquired with a Bruker Avance 500 MHz spectrometer using a ^{1}H−^{19}F−^{15}N−^{13}C 5 mm quadruple resonance inverse (QXI) probe fitted with an actively shielded Z gradient. ^{1}H NMR experiments were performed using presaturation using relaxation gradients and echoes (PURGE) water suppression and 128 scans, a recycle delay of 3 s, and 16 K time domain points.[35] Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum and a zero filling factor of 2, then manually phased and calibrated to the DSS methyl singlet set to a chemical shift (δ) of 0.00 ppm. Metabolite peaks were identified in each spectrum by comparison with previously published assignments.[33,36,37]

Statistical Analyses. ^{1}H NMR spectra between δ of 0.5 and 10 ppm were divided into 0.01 ppm wide buckets, and the area under each segment was integrated with AMIX 3.7.10 (Bruker BioSpin) with the integration mode of sum of intensities. The area in the range δ = 4.70−4.85 ppm was excluded to eliminate small residual H_2O/HOD signals, resulting in a total of 956 buckets for each earthworm. The data were normalized using probabilistic quotient normalization (PQN) to correct for differences in the total NMR signal measured for each sample which can vary with factors such as the mass of tissue extracted.[22,38−43]

Both principal components analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were applied to detect trends in the earthworm metabolic profile related to TiO_2 exposure. PLS-DA models were cross validated using leave-one-out cross validation,[44,45] and the number of components selected for each final PLS model was determined using the 1CV strategy described by Westerhuis et al.[46] For each PLS-DA model, the explained variation of X and Y (R^2X and R^2Y) were reported to indicate how well the model fit the training data,[47] and the internally cross-validated R^2Y value (reported as Q^2Y[48]) was reported as a preliminary measure of the predictive ability of the model.[44,45] In addition, as it has been suggested that misclassification rates provide a better metric for PLS-DA validation than Q^2Y,[49] a permutation test (400-fold permutation of the class labels) was performed to obtain the expected distribution of model sensitivity (i.e., the rate of proper earthworm classification), and this was compared to the observed model sensitivity to assess model significance.[38,47,50]

Statistical significance was assessed at α = 0.05. Means were reported as the mean value ± standard error unless otherwise noted. Multivariate statistics (PCA and PLS) and permutation tests were performed in R[51] using the Chemometrics package.[52]

RESULTS

Principal Component Analysis (PCA) of Earthworm Metabolic Response to TiO_2 Exposure. Initially, PCA was applied to help identify general similarities and differences in the ^{1}H NMR data.[53,54] A PCA of the complete metabolomic data set extracted from all 50 earthworms (10 from each treatment) produced two principal components (PCs) which explained greater than 90% of the total data variance (PCs 1 and 2 explained 74.8% and 16.0% of the total variance, respectively). A plot of mean PC 1 versus PC 2 scores for each treatment suggests that the metabolic profiles of the control earthworms differed from those of the TiO_2 exposed earthworms (regardless of particle size or concentration), as the mean PC scores (±standard error) of all of the TiO_2 exposed earthworms lie outside of the mean PC scores (±standard error) of the control (unexposed) earthworms (Figure 1, SI Figure S3). In addition, the metabolic profile of earthworms exposed to the smaller TiO_2 particles appeared to be distinguished from that of earthworms exposed to the larger TiO_2 particles, especially at the higher (200 mg/kg) TiO_2 concentrations (Figure 1, SI Figure S3). However, the separation related to TiO_2 particle size was less distinct than that between control and TiO_2-exposed earthworms (Figure 1, SI Figure S3).

The PCA loadings identify the regions of the NMR spectra that contribute to the observed differences in PC scores (SI Figure S4). According to the patterns observed in the PC scores plot (Figure 1), metabolites with positive loadings on either PC1 or PC2 should increase in the control earthworms

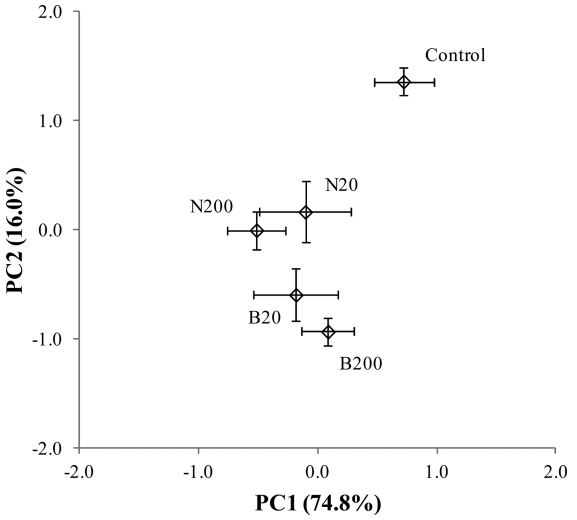

Figure 1. Principal component analysis (PCA) scores plot (PC1 vs PC2) performed using the PQN normalized bucket table for control earthworms and earthworms exposed to soils spiked with either 20 or 200 mg/kg of material N (N20 and N200) or 20 or 200 mg/kg of material B (B20 and B200). Each point represents the mean PC score for the treatment class, and error bars represent the standard error of the mean.

relative to all of the TiO_2 treated earthworms, and metabolites with negative loadings on PC1 or PC2 should decrease in the control earthworms relative to all of the TiO_2 treated earthworms.[47] Negative PC loadings were observed for both PC1 and PC2 for the NMR spectral regions associated with several amino acids (leucine, valine, alanine, glutamate, lysine, tyrosine, and phenylalanine) as well as the organic acid lactate (SI Figure S4). This suggests that all of these metabolites increased in earthworms exposed to both particle sizes of TiO_2 relative to the control earthworms, which is supported by an examination of the percent change in the normalized bucket intensities associated with each of these metabolites (Figure

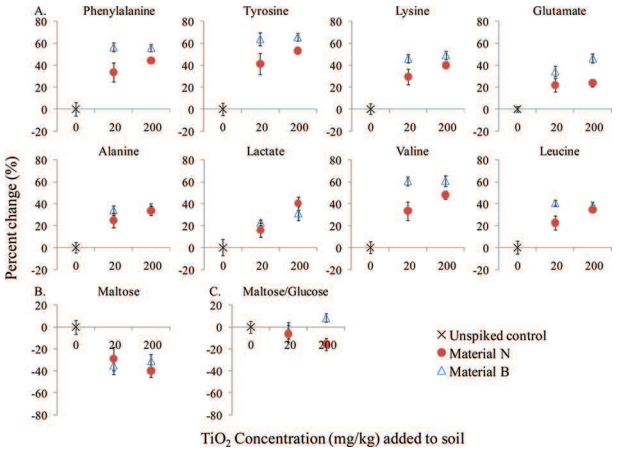

Figure 2. Earthworm metabolite responses to exposure nanosized TiO_2 (material N) and larger-sized TiO_2 (material B) expressed as a percent change from the mean metabolite intensity observed in control (unexposed) earthworms for (a) amino acids, (b) maltose, and (c) overlapping resonances representing both maltose and glucose. Error bars represent standard error of the mean.

2a). For these metabolites, the mean normalized metabolite intensity generally increased by ~15−70% (compared to the control) in the TiO_2-exposed earthworms at both treatment concentrations (20 and 200 mg/kg). The magnitude of this response for the larger-sized TiO_2 (material B) exposed earthworms was generally equal to or greater than that of the nanosized TiO_2 (material N) exposed earthworms, and there was little to no increase for most metabolites between the 20 and 200 mg/kg concentrations regardless of particle size (Figure 2a).

Similarly, positive PC loadings for both PC1 and PC2 were observed for the sugar maltose and for a number of buckets within the region of the NMR spectrum that represents unresolved, overlapping resonances from sugars and amino acids (~3.2−4.2 ppm; SI Figure S4). This indicates that these metabolites decreased in TiO_2-exposed earthworms relative to control earthworms, which concurs with the normalized bucket intensities associated with maltose (Figure 2b). The mean normalized metabolite intensity for maltose decreased in earthworms following exposure to both the 20 and 200 mg/kg concentrations of the TiO_2 materials relative to the control (by ~30−40%). The magnitude of this response was similar for all TiO_2 spiked soils regardless of concentration or particle size (Figure 2b).

A third group of buckets in the loadings plot exhibited a positive PC1 loading and a negative PC2 loading, which suggested that these metabolites increased relative to the control in larger-sized TiO_2 (material B) exposed earthworms and decreased relative to the control in the nanosized TiO_2 (material N) exposed earthworms (SI Figure S4). This group included two buckets that have been identified as representing overlapping resonances between maltose and glucose and a number of buckets that have not been assigned to a specific metabolite but are located within the region of the NMR spectrum that represents unresolved, overlapping resonances from sugars and amino acids (~3.2−4.2 ppm, SI Figure S4). Again, examination of the percent change of the mean normalized bucket intensities supported this interpretation of the loadings plot (Figure 2c). Since earthworms exposed to both sizes of TiO_2 exhibited similar patterns (decreases) for maltose, this difference may indicate a divergence in the response of glucose related to particle size. Specifically, while little to no differentiation from the control was observed for either particle size at the 20 mg/kg concentration, at the 200 mg/kg concentration the metabolites represented by these overlapping resonances appeared to decrease below the control in the nanosized (material N) TiO_2 exposed earthworms (by ~10−15%) and to increase slightly above the control in the larger-sized (material B) TiO_2 exposed earthworms (by ~10%, Figure 2c).

Partial Least Squares Discriminant Analysis (PLS-DA) of Earthworm Metabolic Response to TiO_2 Exposure. Overall, PCA identified patterns in the changes between the metabolic profiles of earthworms from the control, nanosized TiO_2 (material N), and larger-sized TiO_2 (material B) treatments. Therefore, partial least-squares discriminant analysis (PLS-DA) was also performed to determine the significance of the separation between each of the five treatment classes (control, B20, B200, N20, and N200). This supervised analysis created a PLS-DA model with 3 components, an R^2X of 92.9%, an overall R^2Y of 31.3%, and an overall Q^2Y of 16.6%. Although the overall Q^2Y value of this model was low, examination of the Q^2Y and prediction error values for each individual class suggested that membership in certain treatments was predicted better than in other

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Table 2. Model Characteristics for PLS-DA Classification Model Built Using Full Data Set and Results of Significance Testing via 400-Fold Permutation Test

	R^2Y	Q^2Y	sensitivity[a,c]	specificity[b,c]	permutation test results[d]	
					count (n) of sensitivity$_{(perm)} \geq$ sensitivity$_{(obs)}$	p (n/400)
control	0.64	0.56	1	0.88	0	<0.0025
B20	0.15	−0.02	0.2	0.95	171	0.43
B200	0.49	0.33	0.9	0.83	0	<0.0025
N20	0.01	−0.17	0	1	400	1
N200	0.29	0.13	0.6	0.78	1	0.0025
total	0.31	0.17	0.54	0.89	0	<0.0025

[a]Sensitivity: the proportion of earthworms correctly classified as belonging to a class to which they belong. [b]Specificity: the proportion of earthworms correctly classified as not belonging to a class to which they do not belong. [c]A successful classification model will have values approaching unity for both sensitivity and specificity. [d]In order to determine statistical significance of the model, the number of instances for which the permuted sensitivity was greater than or equal to the observed sensitivity for the optimized PLS-DA model was counted (n) and then divided by the total number of permutations (400). The model was considered significant if the generated p value was <0.05.

treatments (Table 2). For example, the Q^2Y ranged from −17% for the N20 treatment group to 56% for the control group (Table 2). Furthermore, permutation test results based on classification errors[49] suggested that this model had enough information to properly classify earthworms in the control, B200, and N200 treatments ($p < 0.0025$) but performed poorly at classifying the earthworms in the B20 and N20 treatments ($p > 0.43$, Table 2). In addition, by examining the specific misclassifications which occurred during the cross-validation process, it was observed that 4 of the 10 N200 earthworms were wrongly classified as either B20 or B200, which suggests a moderate amount of overlap between the metabolic profiles of earthworms exposed to both sizes of TiO_2 with each other. In general, these findings match well with the sample distributions previously demonstrated in the PCA scores plot (Figure 1, SI Figure S1).

DISCUSSION

The results of the PCA and PLS-DA analysis suggests that earthworms exhibited significant changes (relative to the untreated control) in their metabolic profile following exposure to the higher concentration (200 mg/kg) of TiO_2 in soil for both of the studied particle sizes. These changes reflect significant alterations in the underlying network of earthworm metabolic pathways. As is frequently the case for sublethal biomarkers, it is difficult to conclusively determine whether this response indicates an adaptive or adverse response.[55−57] However, as previous studies have demonstrated a correlation between metabolomic responses and ecologically relevant end points (e.g., cocoon production rate in earthworms[20] and scope for growth in marine mussels[25]), the possibility that this may indicate a stress response in earthworms to TiO_2 materials should be considered.

The observed metabolomic response (increased alanine and other amino acids and decreased maltose) for TiO_2 exposed earthworms (for both sizes of TiO_2 material) is similar to that previously observed for earthworms exposed to phenanthrene.[17−19] This may suggest that this pattern of metabolomic response represents a toxic MOA common to phenanthrene and both particle sizes of TiO_2. Previous studies have suggested that nanosized TiO_2 can initiate oxidative stress via formation of reactive oxygen species which can damage lipids, carbohydrates, proteins, and DNA.[11,58,59] This is supported by other studies which have observed antioxidant enzyme responses to nanosized TiO_2 exposure in various organisms,[59−61] including the earthworm *E. fetida* used in the current study.[11] The same antioxidant enzyme responses have also been observed in various organisms (including *E. fetida*) following exposure to phenanthrene[62] and other organic contaminants including pyrene, the organophosphate pesticide fenitrothion, and explosive compounds [e.g., 1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)] for which the proposed toxic MOA is also oxidative stress.[63−65] This may explain why these very different compounds induce similar patterns of metabolomic response in *E. fetida*. This hypothesis is strengthened by a previous study by Forcella et al.[66] which observed both increased antioxidant enzyme activities and similar metabolic changes (increased alanine and lactate and decreased trehalose sugar concentrations) in the aquatic invertebrate *Chironomus riparius* following exposure to fenitrothion. However, as specific markers of oxidative stress (e.g., measurements of antioxidant enzyme activity) were not quantified in this experiment, this link cannot be conclusively demonstrated.

As the toxicity of nanosized materials is hypothesized to be linked to both their small size and consequently increased relative surface area,[4,67,68] it was expected that material N (mean diameter 20 nm, Table 1) would elicit a greater metabolic response in earthworms than material B (mean diameter 118 nm, Table 1). Earlier studies supporting this hypothesis demonstrated significant decreases in various markers of toxicity (including survival, reproduction, and markers of oxidative stress) following exposure to micrometer-sized relative to nanosized (≤50 nm) TiO_2 for species such as zebrafish,[69] lugworms,[58] and nematodes.[70] However, the earthworm metabolic responses observed in the current study were very similar for both sizes of TiO_2 materials used (Figures 1and 2). Although earthworms exposed to both sizes of TiO_2 materials were well separated from the control in both the PCA and PLS-DA analyses (Figure 1, SI Figure S1 and Table S2), there was a notable amount of overlap in metabolic profiles of earthworms exposed to materials N and B (Figures 1 and 2, SI Figure S1 and Table S2). This suggests that the relative toxicity of TiO_2 materials to *E. fetida* may not be governed solely by primary particle size.

It is difficult to compare the findings of the current study regarding the influence of TiO_2 particle size on earthworm toxicity to previous research due to variations in the specific TiO_2 materials used in each study. Hu et al.[11] reported that *E. fetida* exhibited increased antioxidant enzyme activity, DNA damage, and mitochondrial damage following a seven day exposure to nanosized TiO_2 (diameter 10−20 nm) at concentrations greater than or equal to 1000 mg/kg in soil

(responses were not significant at lower concentrations). However, Hu et al.[11] only studied one particle size, so it is unclear whether this toxic response was size-dependent. In contrast, Heckmann et al.[12] did note an apparent size-related difference in TiO_2 toxicity to *E. fetida*; exposure to nanosized TiO_2 at a concentration of 1000 mg/kg in soil for 28 days significantly impaired reproduction, but exposure to larger-sized TiO_2 materials did not. However, Heckman et al.[12] did not provide a detailed characterization of the larger sized TiO_2 material used as a control in their study. For the nanosized material in their study, they characterized and reported the primary particle size (30 nm diameter) and crystallinity (73% anatase and 27% rutile); however, for the bulk control only the nominal diameter (40 μm) was reported, and no information regarding the crystal phase of the material was given.[12] As it has been suggested that the crystal phase of TiO_2 may be more important than particle size for predicting toxicity,[68,72] it is possible that a difference in the crystallinity of the two TiO_2 materials used may have significantly confounded their results regarding the role of particle size.

Of the two main industrially relevant crystal phases of TiO_2 (anatase and rutile), evidence suggests that the anatase form used in the current study may be more toxic.[73] Even at larger sizes (up to 195 nm diameter), anatase TiO_2 has demonstrated potential to generate reactive oxygen species.[68] In addition, Adams et al.[71] noted that anatase TiO_2 materials with mean measured particle diameters of between 300 and 1000 nm significantly impaired growth in both Gram-positive (*Bacillus subtilis)* and Gram-negative (*Escherichia coli*) bacteria, with no apparent reduction in toxicity related to increased particle size. This demonstrates that although micrometer-sized TiO_2 materials (>100 nm) are generally considered nontoxic,[1] they can generate toxic responses in certain living organisms, at least when anatase phase materials are considered. This could explain the observed earthworm response to the larger sized materials in the current study.

A parallel study using the same TiO_2 materials (with materials N and B labeled as materials A and E) reported that neither material significantly impaired *E. fetida* survival (14 d), reproduction (56 d), or juvenile growth (18 w) at the same concentrations (20 or 200 mg/kg) used in the current study.[27] Even at concentrations as high as 10 000 mg/kg, neither material significantly impaired reproduction (56 d) of *E. fetida* or of the related earthworm *E. andrei*.[27] The only significant earthworm response noted by McShane et al.[27] was avoidance (48 h) of soils spiked with ≥1000 mg/kg of material N by the earthworm *E. andrei*. Therefore, it is clear that these materials are not acutely toxic to earthworms. However, the results of the current study demonstrate a significant alteration in earthworm metabolic pathways following exposure to these materials, which may be indicative of subtle, sublethal stress. As earthworms have multiyear lifespans,[74] it is important to consider what these sublethal responses might represent and whether they could lead to organism or population level toxicity during chronic environmental exposures. This could be explored by applying more targeted molecular and cellular level ecotoxicological end points to determine the exact nature of the response and/or through the application of multigeneration tests.

Overall, the results of this study suggest that earthworms exhibited significant changes in their metabolic profile following TiO_2 exposure for both of the studied particle sizes and that this response was less variable when the TiO_2 concentration was increased from 20 to 200 mg/kg. The observed earthworm metabolic response to both TiO_2 materials may be indicative of oxidative stress, which has been proposed as a toxic MOA related to nanomaterial exposure in a variety of organisms including earthworms,[11,58,59] but this must be confirmed by further, more targeted testing. In addition, metabolomics may be a more sensitive method for the detection of earthworm sublethal response to TiO_2 in soil than traditional reproductive parameters. This highlights the value of applying nontargeted metabolomics to assess the sublethal response of an organism to an emerging contaminant with an unknown MOA. As metabolomics measures the end products of multiple metabolic pathways simultaneously and in a nontargeted fashion, it may be able to detect organism sublethal responses to contaminants at much lower concentrations than traditional ecotoxicological end points.[20,23−25]

■ ASSOCIATED CONTENT

Supporting Information

Additional information regarding TiO_2 material characterization. Figures S3 and S4 providing more information on the PCA analysis shown in Figure 1 and Table S2 listing the classification results of individual earthworms following leave-one-out cross-validation. This material is available free of charge via the Internet at http://pubs.acs.org.

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