Toxicogenomic Analysis Provides New Insights into Molecular Mechanisms of the Sublethal Toxicity of 2,4,6-Trinitrotoluene in Eisenia fetida

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Toxicogenomic Analysis Provides New Insights into Molecular Mechanisms of the Sublethal Toxicity of 2,4,6-Trinitrotoluene in *Eisenia fetida*

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Xenobiotics such as explosives and pesticides released into the environment can have lethal and sublethal impacts on soil organisms such as earthworms with potential subsequent impacts at higher trophic levels. To better understand the molecular toxicological mechanisms of 2,4,6-trinitrotoluene (TNT), a commonly used explosive, in *Eisenia fetida*, earthworms were exposed to a gradient of TNT-spiked soils for 28 days and impacts on gene expression were examined using a 4032 cDNA microarray. Reproduction was increased at low doses of TNT, whereas high doses of TNT reduced juvenile production. On the basis of reproduction responses to TNT, four treatments, that is, control, 2, 10.6, and 38.7 mg/kg, were selected for gene expression studies in a balanced interwoven loop design microarray experiment in which the expression of 311 transcripts was significantly affected. Reverse-transcription quantitative polymerase chain reaction (RT-QPCR) data on 68 selected differentially and nondifferentially expressed transcripts showed a significant correlation with microarray results. The expression of genes involved in multiple biological processes was altered, including muscle contraction, neuronal signaling and growth, ubiquitinylation, fibrinolysis and coagulation, iron and calcium homeostasis, oxygen transport, and immunity. Chitinase activity assays confirmed down-regulation of chitinase genes as indicated by array and RT-QPCR data. An acute toxicity test provided evidence that dermal contact with TNT can cause bleeding, inflammation, and constriction, which may be explained by gene expression results. Sublethal doses of TNT affected the nervous system, caused blood disorders similar to methemoglobinemia, and weakened immunity in *E. fetida.*

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Introduction

The subclass Oligochaeta (commonly known as "earthworms") of the Annelida phylum has more than 8000 terrestrial and aquatic (both freshwater and marine) described species (*41*). Earthworms are enormously important in the construction and fertility maintenance of the soil, making them one of the best indicators of soil quality. Some earthworm species are used as ecotoxicological model organisms for which both acute and reproductive toxicity tests have been standardized (*40*). Although there is a large database of earthworm toxicity tests, these tests evaluate only a few descriptive endpoints such as lethality, weight change, and cocoon or juvenile counts. In addition, these bioassays are lengthy (14–56 days) and do not provide mechanistic insight for the test compounds.

Historical and continued production and use of munitions has resulted in the contamination of military-related lands and facilities. Unexploded or partially exploded ordnance on these sites can release munitions constituents such as 2,4,6-trinitrotoluene (TNT) into the soil (*22*). Human and animal health effects of TNT include anemia, abnormal liver function, skin irritation, and cataracts (*3, 23, 24, 39*). Since hundreds or thousands of milligrams per kilogram of TNT residues can be found in surface soils (*22*), earthworms are ideal species to study the impact of TNT on the environment.

As a producer of reactive oxygen (ROS) or nitrogen (RNS) species (*9, 25–27*), TNT may target nucleic acids, proteins, and lipids, directly causing cell injuries or disrupting signal transduction (*6*). TNT has been found to induce damage to spermatozoa in male rats through oxidative DNA damage mediated by its metabolite, 4-hydroxylamino-2,6-dinitrotoluene (*18*). Cytotoxicity, genotoxicity, lethality, and reproductive toxicity of TNT have also been reported in various organisms and cell lines (see reviews (*21, 28, 46, 54*)). However, few studies have related gene expression changes to TNT toxicity (*14*). In the present study, we investigated the mode of action by which TNT causes toxicity in *Eisenia fetida* using a toxicogenomic approach.

Previously, we used suppressive subtractive polymerase chain reaction (PCR) to isolate 4032 *E. fetida* cDNA clones affected by metals and nitroaromatic and nitroheterocyclic compounds (*37*). The specific objectives of this work were to screen these clones to (1) identify genes affected by exposure to TNT, (2) identify or predict toxicological modes of action for TNT based on gene expression, and (3) generate new hypotheses of biological pathways involved in response to TNT exposure. Our ultimate goal is to elucidate the molecular mechanisms of TNT toxicity in *E. fetida.*

Materials and Methods

Animal Culture and Chemicals. *E. fetida* were maintained in a continuous culture from stocks obtained from Carolina Biological Supply Company (Burlington, NC; see Supplementary Methods in the Supporting Information). Fully clitellate adults weighing 0.3–0.6 g (live weight) were selected for all experiments. TNT (CAS no. 118-96-7, purity > 99%) was purchased from Chem Service (West Chester, PA).

Earthworm Toxicity Test. Reproductive toxicity tests were conducted in a field-collected silty loam soil (3% sand, 72% silt, 26% clay; pH 6.7; total organic C 0.7%, and CEC 10.8 mEq/100 g) amended with TNT at 0, 0.6, 1.1, 2.0, 3.4, 6.2, 10.6, 13.6, 20.8, 38.7, and 67.1 mg/kg of soil to establish an exposure effect relationship between TNT and juvenile and cocoon counts in accordance with standard guidelines (*2*). Further details on the soil spiking and toxicity test can be

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found in the Supplemental Methods (Supporting Information). Briefly, each TNT treatment had five replicate jars with five mature worms and 250 g (dry weight equivalent) of soil in each replicate. Adult worms were removed, counted, and weighed at 28 days with one worm per replicate reserved for gene expression in RNAlater solution (Ambion, Austin, TX) at –80 °C, and the remaining worms were snap-frozen at –80 °C until being used for chitinase activity assays.

Microarray Experiments. Gene expression was examined in five worms at each of four treatments, 0 (solvent control), 2.0, 10.6, and 38.7 mg TNT/kg soil. Samples at these TNT concentrations were examined as they represent an exposure causing an increase in juvenile production, a midrange exposure with no effect on juvenile production, and a high exposure with a decrease in juvenile production. Microarray analysis was performed using mRNA separated from total RNA using a Qiagen RNeasy Mini Kit (Valencia, CA) followed by a NucleoTrap Nucleic Acid Purification Kit (BD Biosciences, Franklin Lakes, NJ). Microarray probes were synthesized from 30 ng of mRNA combined with SpotReport Alien mRNAs (Stratagene, La Jolla, CA) and labeled with Cy3 or Alex Fluor 647 dye using 3DNA 900 Expression Array Detection kits (Genisphere, Hatfield, PA). SpotReport Alien mRNAs were used as spike-in controls to monitor array analysis efficacy as recommended by the manufacturer.

Probes were hybridized to glass slide arrays printed with 8704 features including 60 Stratagene SpotReport Alien cDNA spots (complimentary with Alien mRNAs), 84 water spots, 256 blank spots, and 240 printing buffer spots in addition to 4032 cDNA clones (twice) that were isolated from the same continuous culture used for TNT exposures reported in this work (*37*). A balanced interwoven loop design was chosen for hybridization where each of the five biological replicates per four doses were hybridized four times on four different arrays including two dye label swaps (*11*) (Figure S1, Table S1, Supporting Information). Arrays were scanned using a 5 μm resolution VersArray ChipReader (Bio-Rad, Hercules, CA) and processed using VersArray Analyzer Software v. 4.5 (Bio-Rad) with deposition of all data into the GEO database (serial number GSE7024).

Microarray Data Analysis. Spot intensity data were filtered to remove those points that were below background, overlapping, or oversaturated. The filtered data were background subtracted and normalized by cross-channel LOWESS (local regression) and centering to each channel's mean spot intensity. We applied two different statistical algorithms in the analysis of array data based on our observations that identification of differentially expressed genes is algorithm-dependent and the convergence of data sets from different algorithms gives a list of genes to examine that is more conservative and where the choice of each method is validated by the other. Therefore, we used BAGEL (Bayesian Analysis of Gene Expression Levels) and SAM (Statistical Analysis of Microarrays) to derive a common list of differentially expressed genes (*47, 48*). The use of two statistical programs based on different algorithms should theoretically increase the inference stringency and reduce false positives. Details of the statistical analysis of array data can be found in the Supplemental Methods (Supporting Information).

Reverse-Transcription Quantitative PCR (RT-QPCR). SYBR Green RT-QPCR was used to confirm the expression of selected transcripts in the same mRNA samples as used in microarray hybridization. Starting mRNA concentrations and PCR efficiencies were calculated using LinRegPCR (*38*). The mRNA concentrations were normalized to the geometric mean of the three most stable genes using geNorm v. 3.4 (*51*). Details on primer sequences, protocols, and data analysis are given in the Supplemental Methods, Table S2, and Figure S2 (Supporting Information).

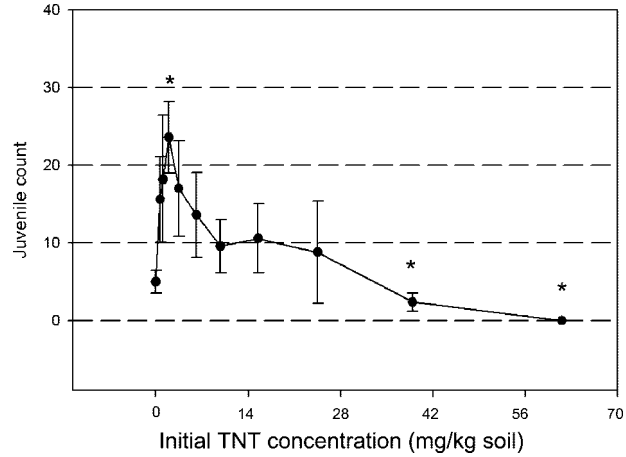

FIGURE 1. Dose-response curve of earthworm reproduction toxicity test. Juvenile counts were made at the termination of the test (day 56). Error bars represent standard error ($n = 5$). No juveniles were found in the soil with the highest TNT concentration. An asterisk (*) indicates a significant difference from the control ($p < 0.05$, two-tailed *t*-test).

Filter Paper Contact Test. A separate contact test was performed to further observe the earthworm physiological response to TNT by dermal exposure. Details of this test can be found in the Supplemental Methods (Supporting Information). Briefly, individual worms were exposed in glass vials to moistened filter paper containing TNT. The concentrations of TNT were 0, 0.94, 1.7, 2.9, 4.6, 7.7, 13, 22, 36, 60, and 100 μg/cm^2 in 10 replicate vials. Worms were observed over 4 days for visually detectable adverse effects.

Chitinase Activity. Chitinase enzyme activity was measured as described in ref *8* in replicate worms exposed for 28 days in soils spiked with TNT at 0, 2, 10.6, and 38.7 mg/kg to confirm array and RT-QPCR results (see Supplemental Methods, Supporting Information, for details).

Results

The Effect of TNT on Earthworm Reproduction. The 28 day exposure to TNT caused lethality only at the two highest concentrations. At 67.1 mg/kg of TNT, 40% of the worms were dead on day 28 ($p < 0.05$, two-tailed *t*-test against the control). There was no significant difference in growth (fresh weight change of surviving worms). Growth was an insensitive endpoint as healthy worms often lose weight due to high offspring outputs. The two reproduction endpoints, juvenile counts (Figure 1) and cocoon counts (data not shown), were significantly affected by TNT. As observed in our previous unpublished studies, TNT exposure induced hormetic effects at low levels (<10.6 mg/kg soil) that peaked at 2 mg/kg of soil (Figure 1). Reproduction decreased significantly at 38.7 mg/kg and higher.

Effect of TNT on Gene Expression. Bayesian analysis of array data identified 572 cDNAs as differentially expressed between the control and the TNT-treated worms, while SAM inferred 472 significant transcripts (false discovery rate = 3.6%) with 311 transcripts (42% of the 733 nonredundant significant cDNAs) identical between the two lists. Of the 311 common transcripts, 154 had significant blastx matches in the GenBank nonredundant database with an Expect value $<10^{-4}$, putatively coding for ca. 74 unique genes (Figure 2; Table S3, Supporting Information).

We selected 68 transcripts putatively encoding 16 different genes to validate the relative expression results obtained from microarray experiments. Among the 68 transcripts, five were nonsignificant transcripts, six were identified as significant by either SAM or BAGEL, and 57 were identified as significant by both programs. A fairly good agreement exists between

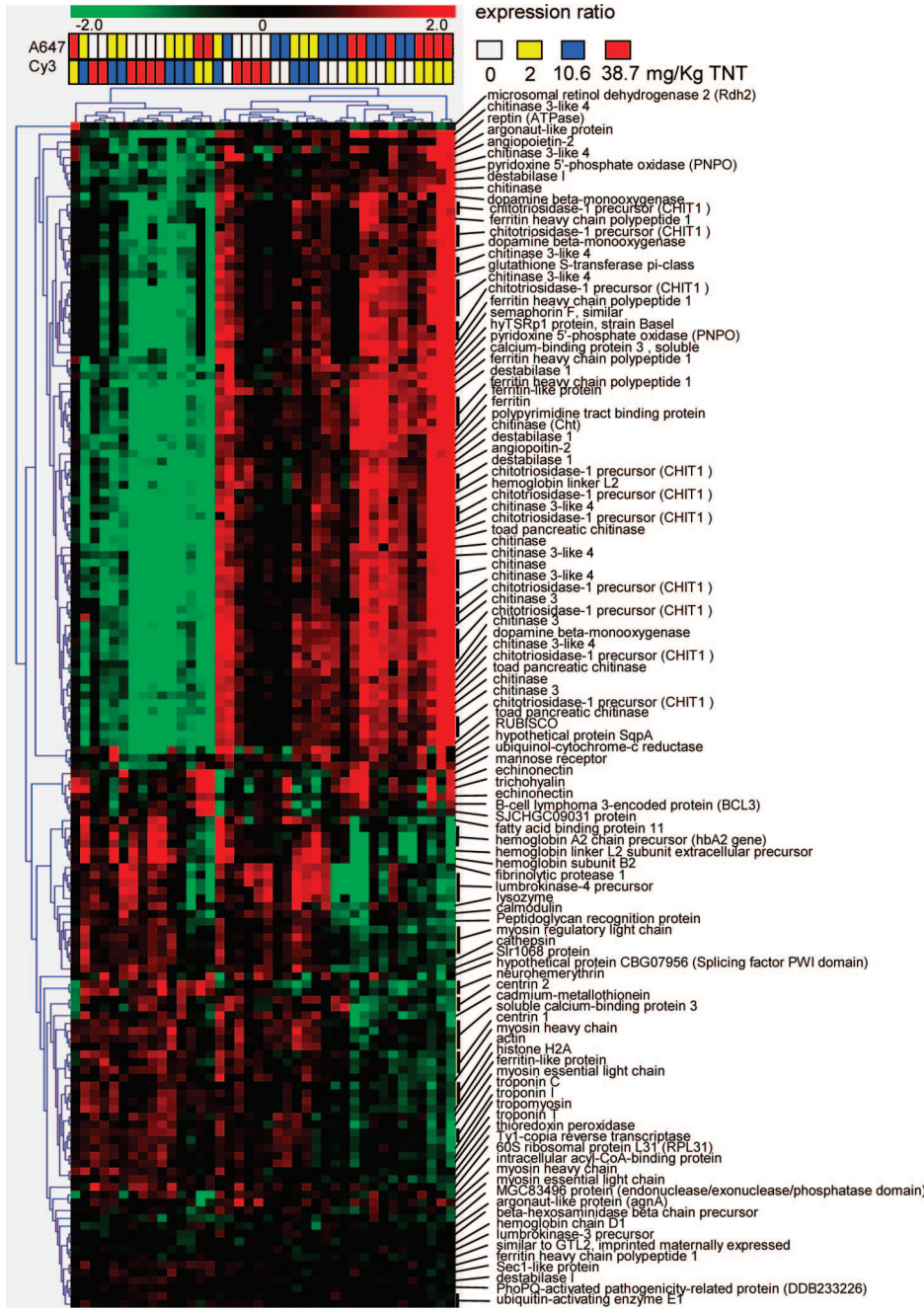

FIGURE 2. Clustering of 154 significant differentially expressed transcripts with a blastx Expect value $<10^{-4}$ in earthworms exposed to TNT. Expression heat maps of ratio data for all 40 hybridization experiments were compared using hierarchical clustering of sample hybridizations on the *x* axis and gene expression values on the *y* axis.

microarray and RT-QPCR results despite some potential differences due to different assay sensitivities and data normalization, transformation, and statistical methods used (Table 1). The correlation between mean values of the two data sets is statistically significant ($n = 75$ excluding the control treatment, $r = 0.74$, $F = 85$, $p < 0.001$). Particularly noticeable, as shown by both microarray and QPCR results, transcripts coding for chitinase and ferritin were consistently down-regulated while those coding for centrin 1 and myohemerythrin up-regulated at 38.7 mg/kg of TNT. A number of genes (e.g., fatty acid binding protein 11) were up-regulated at 2 mg/kg corresponding to the hormetic peak seen for the reproduction endpoint (Table 1; Figure 1).

Contact Toxicity of TNT. We performed a filter paper contact test to further explore the development of mortality as well as the manifestation of sublethal effects as predicted by genomic analysis because soil exposure tests do not facilitate such observations. Worms exposed to TNT on filter paper showed dose- and time-dependent lethal and sublethal effects (Figure 3A). Mortality occurred at 4.6 μg/cm^2 and higher. The predominant effects included inflammation, coelomocyte excretion, and bleeding (Figure 3B). We also observed rigidity similar to that previously reported in worms exposed to neurotoxicants (*17*), suggesting that TNT may affect worm nervous systems as well. Constriction was often observed in worms 1 $\sim$ 2 days prior to death (Figure 3B). Exposure to doses $\geq$13 μg/cm^2 resulted in immediate stress responses such as coelomocyte excretion and inflammation, potentially an indication of dermal irritation due to TNT. Dermatitis has also been observed in human TNT intoxication (*54*). During the 4 day dermal contact exposure, a number

TABLE 1. Comparison of Array and RT-QPCR Mean Expression Results for Selected Genes

		Microarray			RT-QPCR		
Putative gene name	Primer set #	2 ppm	10.6 ppm	38.7 ppm	2 ppm	10.6 ppm	38.7 ppm
Cytochrome oxidase subunit II	1	0.99	0.90	1.07	1.15	0.97	1.09
NADH dehydrogenase 1 β	2	1.05	1.11	1.21	1.11	0.91	0.99
Centrin 1	3	0.89	1.15	1.41	0.93	1.35	1.58
Centrin 2	4	0.68	0.81	1.14	0.93	0.88	0.77
Lumbrokinase	5	0.82	1.29	1.67	0.97	1.44	1.39
	6	0.89	1.22	1.67	0.96	1.15	1.08
Metallothionein	7	1.00	1.02	1.15	0.96	0.74	1.51
Myohemerytherin	8	1.15	1.35	1.61	1.17	1.35	1.25
ADP ribosylation factor	9	1.15	0.96	0.95	0.95	0.99	1.10
Serine-type endopeptidase inhibitor	10	1.62	0.91	1.15	1.55	0.99	1.05
Macrophage mannose receptor	11	1.57	0.97	0.79	1.34	0.94	0.64
Chitinase	12	0.92	0.66	0.43	1.28	0.76	0.63
	13	1.11	0.64	0.48	1.35	0.91	0.88
	14	1.02	0.57	0.43	1.49	0.80	0.67
	15	1.06	0.65	0.46	1.19	0.66	0.56
	16	1.11	0.68	0.43	1.62	0.81	0.58
	17	1.10	0.64	0.49	1.51	0.81	0.52
	18	0.89	0.57	0.46	1.27	0.71	0.71
Ferritin	19	1.19	0.64	0.51	1.36	0.60	0.63
	20	1.01	0.53	0.42	1.44	0.69	0.43
	21	1.13	0.69	0.43	1.61	0.64	0.48
Dopamine β-monooxygenase	22	0.89	0.61	0.53	1.39	0.78	0.81
18S rRNA	23	1.00	0.94	0.96	0.62	1.10	0.90
Fatty acid binding protein 11	24	0.60	1.01	1.21	0.54	1.00	0.99
Actin	25	0.94	1.22	1.18	0.94	1.09	0.84

[a] Color-highlighted expression values are statistically different from the control, with green representing down-regulated whereas red represents up-regulated genes. Values are relative to control samples. See Table S2, Supporting Information, for primer sequences and their target clones. Table S4, Supporting Information, contains 97.5% upper and lower confidence boundaries for microarray data and standard errors for RT-QPCR data.

of worms died as a consequence of severe bleeding, inflammation, and constriction.

Effect of TNT on Chitinase Enzyme Activities. To validate the down-regulation of several chitinase genes, we assayed chitinase activity against three different chitinase substrates in worms from the same treatment groups used for microarray experiments. Chitinase activity targeting the substrates 4-methylumbelliferyl β-D-N,N′-diacetylchitobioside for chitobiosidase activity and 4-nitrophenyl N-acetyl-β-D-acetylglucosaminide for β-N-acetylglucosaminidase activity was not significantly different between the four treatments (data not shown). However, endochitinase activity was significantly reduced in all three of the TNT treatments as compared to controls (Figure 4).

Discussion

Although little effect on mortality and growth was observed in adult worms after 28 days of exposure to up to 38.7 mg TNT/kg soil, TNT exposure did impact reproductive endpoints (Figure 1) and altered the expression of genes that can be related to a diverse range of functions (Figure 2; Table S3, Supporting Information). TNT and its hydroxylamino and amino metabolites have been shown to form ROS and RNS

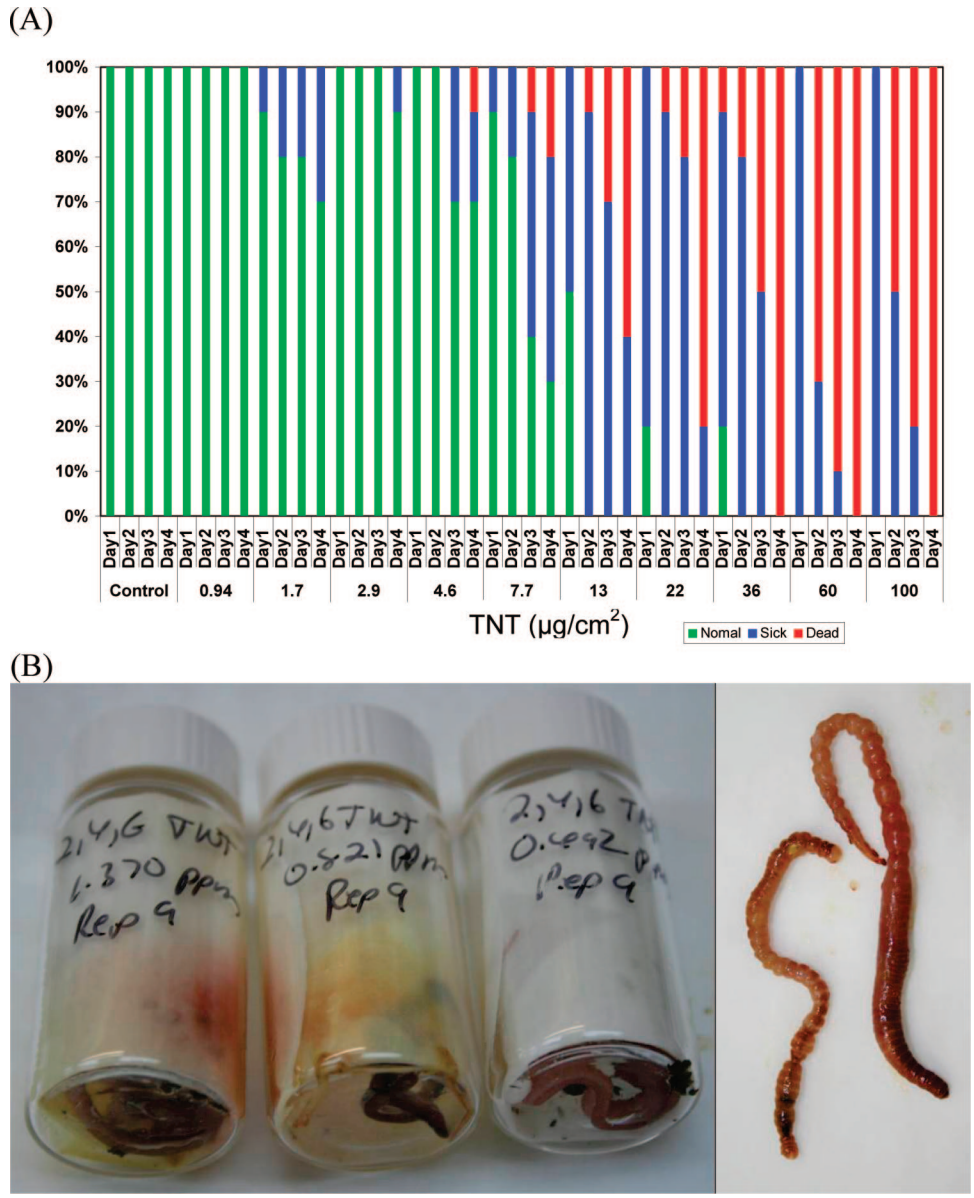

FIGURE 3. Effect of 4 day exposure to 2,4,6-TNT on the earthworm *Eisenia fetida* in a filter paper contact test. (A) The percentage of normal, sick, and dead worms ($n = 10$) as a function of TNT dosage and length of exposure. Worms were recorded as being sick if any of the three symptoms was observed, that is, coelomocyte excretion, inflammation, and bleeding. Worms were determined dead if autotomy/constriction or motionlessness after repeated poking was observed. (B) Shown in the left panel are three worms exposed to filter paper treated respectively with 7.7 (labeled 0.492 ppm), 13 (0.821 ppm), or 22 (1.370 ppm) μg/cm^2 of TNT. Red-colored filter paper indicates worm bleeding. The right panel shows the constriction of another worm treated with 13 μg/cm^2 of TNT.

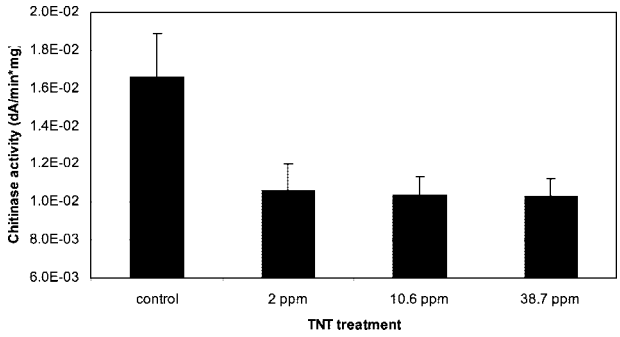

FIGURE 4. Endochitinase activity in earthworms as affected by 28 day TNT exposure. The columns represent the mean of five replicates and the error bars the standard error.

through nitro side-group redox cycling by neuronal nitric oxide synthase (*26*), hemoglobin (*30*), ferredoxin/NADP(+) reductase, and other nitroreductases (*34*). Changes in the expression of metallothionine (*16*), thioredoxin peroxidase, glutathione-s-transferase, and catalase indicate that TNT-exposed worms were under oxidative stress (Figure 5). The reduced fecundity at 38.7 mg of TNT/kg of soil may be attributed to multiple modes of action identified by genomic analysis, including oxygen transport, antioxidant response, muscle contraction, neurological function, calcium binding and signaling, and immune function (Figure 5) as TNT appears to act on various targets including nucleic acids, proteins, lipids, and signaling pathways (*6*).

Oxygen Transport and Iron Homeostasis. A predominant effect of TNT exposure in animals is methemoglobinemia resulting from the oxidation of hemoglobin (*3, 39, 54*). Our sequencing results indicate that *E. fetida*, like other annelids, possesses two completely different types of oxygen-binding proteins: hemoglobins in the blood and hemerythrins in the vascular system and the coelomic fluid or in muscles

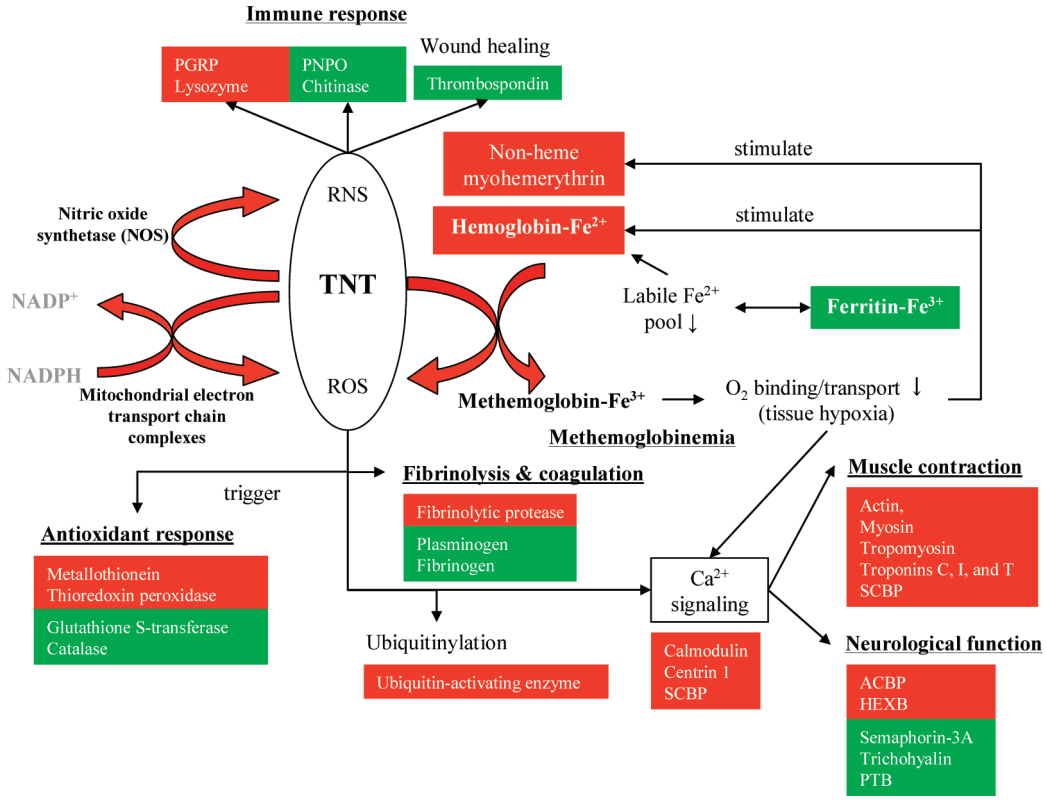

FIGURE 5. Sublethal effects of TNT on earthworms reflected as significant gene expression changes observed in the present study. Genes in red-colored boxes are up-regulated, and green-colored boxes are down-regulated at 10.6 or 38.7 mg/kg of TNT. Acronyms: ROS = reactive oxygen species; RNS = reactive nitrogen species; PGRP = peptidoglycan recognition protein; PNPO = pyridoxine-5-phosphate oxidase; SCBP = soluble calcium binding protein; ACBP = acyl-CoA-binding protein; HEXB = β-hexosaminidase beta chain; PTB = polypyrimidine tract binding protein.

(myohemerythrins). Both types of proteins can reduce TNT via one-electron reduction to produce their met forms (*9, 30*). Continued oxidation by TNT can create tissue hypoxia as the met forms cannot bind and transport oxygen. In TNT-exposed earthworms, we observed that transcripts coding for hemoglobin (subunits A2, B2, and D1 and linker L2) and myohemerythrin were up-regulated, suggesting a compensatory response to increase oxygen-binding and -transport capacity. We also observed down-regulation of the Fe^{3+} storage protein ferritin, potentially due to an increased Fe^{2+} demand as a result of excessive oxidation of Fe^{3+} and depletion of the labile Fe^{2+} pool. We have observed similar impacts on gene expression in livers of fathead minnows where hemoglobin and iron transport increased with increasing exposure to 2,4-dinitrotoluene (*53*).

Blood Coagulation and Fibrinolysis. TNT exposure affected many genes involved in the blood coagulation cascade and fibrinolysis pathways potentially as a result of the release and buildup of free heme in cells due to TNT oxidation of the hemoglobin, ultimately leading to inflammation and bleeding as observed in filter paper contact tests (Figures 3 and 5). Similarly, hemolytic anemia has been reported in TNT-exposed vertebrates (*23, 24, 39, 54*). Consistent with these observations, expression of fibrinolytic protease, fibrinogen, and plasminogen was affected by TNT exposure (Figure 5). Consequently, various biological processes involving the conversion of fibrinogen to fibrin may be affected, including coagulation, wound healing, fibrinolysis, inflammation, and angiogenesis (www.ebi.ac.uk/interpro/potm/2006_1/Page1.htm).

Neurological Dysfunction. Oxidative stress is closely associated with neurological dysfunctions (*1*). In this study, several lines of evidence indicate that TNT treatment altered the expression of genes related to neural function and growth, such as the acyl-CoA-binding protein which is involved in modulation of GABAergic transmission (*31*); the β-hexosaminidase β chain which is responsible for the degradation of ganglioside GM2 in neurons (*7*); semaphorin-3A, trichohyalin, and polypyrimidine tract binding proteins which play important roles in neuronal growth and differentiation (*29, 43, 49*); and ubiquitin-activating enzyme which is involved in ubiquitinylation or ubiquitin-mediated protein degradation (*35*). Collectively, the altered expression of these genes suggests that neuronal function may be impaired in earthworms exposed to TNT.

Calcium Binding and Signaling. Several transcripts related to genes involved in calcium binding, transport, and signaling were up-regulated in TNT-exposed worms, including genes coding for soluble calcium binding protein (SCBP), calmodulin (CaM), and centrins. This suggests that TNT may disrupt calcium homeostasis and signaling pathways. As an intracellular messenger, Ca^{2+} signals control a diverse array of processes from fertilization and development to apoptosis (*5*). TNT may dramatically enhance ROS production through NADP–NADPH redox cycling by interacting with electron transport chain complexes in the inner mitochondrial membrane, thereby affecting Ca^{2+} homeostasis (*6*). Evidence in mammalian systems also indicates that oxidative stress may decrease the ability of oxidized CaM to bind, activate, and regulate the interactions of Ca^{2+}/CaM-dependent protein kinase II, leading to a reduction in oxidative repair mechanisms (*42*). Disruption of calcium homeostasis can eventually cause cell death via apoptosis or necrosis with net effects being neuronal diseases (*12*).

Muscle Contraction. A suite of genes related to muscle contraction and relaxation, actin, myosin, tropomyosin (Tm), troponin C (Tnc), troponin I (TnI), troponin T (TnT), and SCBP, are impacted by TNT exposure. Contraction of striated

muscle is regulated by a Ca^{2+}-sensitive switch that is located on actin filaments and composed of four proteins: Tm, TnT, TnI, and TnC. It is widely accepted that TnT tethers TnI and TnC to the thin filament via Tm and that TnT/Tm regulates the activation and inhibition of the myosin–actin interaction in response to changes in intracellular Ca^{2+} (*32*). Like parvalbumin in vertebrates, SCBP may regulate muscle relaxation after contraction and prevents nonmuscle cells, particularly neurons, from calcium-induced cell damage through facilitating the diffusion of Ca^{2+} ions and inhibiting the ATPase of isolated body-wall muscle actomyosin (*4, 20*). Changes in calcium homeostasis and cascade signaling pathways suggested by gene expression analysis are likely to be involved in affecting the body-wall muscle contraction and rigidity observed in filter paper contact tests (Figure 3).

Impaired Immune Function. Innate immunity is an evolutionarily ancient system that provides organisms with defense mechanisms against a wide variety of pathogens without requiring prior exposure (*52*). Innate immunity in earthworms is maintained by coelomocytes or leukocytes (*13*). The expression of peptidoglycan recognition protein and lysozyme genes involved in recognition and innate immune defense against bacteria (especially Gram-positive bacteria) (*13, 45*) was up-regulated, potentially as a result of increased Gram-positive bacteria in TNT-contaminated soil (*15*).

As a nonchitinous organism, worm phagocytes can produce and release the highly conserved chitinase as a component of innate immunity against chitin-containing pathogens because chitinase is involved in cell-wall chitin metabolism and catabolism (*50*). More than 10 transcripts similar to human phagocyte-derived chitotriosidase were suppressed in TNT-exposed worms along with two other chitinase isoform genes. Our enzyme assays demonstrate that *E. fetida* possesses chitobiosidase, exochitinase, and endochinase activities, all of which are required to efficiently break down chitin, and that TNT treatment reduced endochitinase activity, confirming gene expression results.

The expression of two transcripts putatively encoding pyridoxine-5-phosphate oxidase (PNPO), a key flavoprotein that catalyzes vitamin B6 synthesis (*33*), was suppressed at both 10.6 and 38.7 mg of TNT/kg. PNPO is especially important in maintaining healthy nerve and muscle cells, and for the production of erythrocytes and leukocytes (*19*). The effects of vitamin B6 deficiency on human immunologic activities include impaired antibody production and declined lymphocyte proliferation (*44*). Decreased expression of PNPO and chitinase, suggesting an immune system weakened by TNT, is consistent with our previous observations that 21 days of exposure to a TNT-spiked soil caused a significant decrease in the ability of worms to reduce nitroblue tetrazolium (*36*), a nonspecific immunotoxicity biomarker (*10*).

In summary, we used a toxicogenomics approach to study molecular mechanisms involved in the sublethal toxicity of TNT in *E. fetida*. Some of the differentially expressed genes (e.g., centrin 1, metallothionein, myohemerytherin, chitinase, and ferritin) are potential candidates for novel biomarkers, for which further screening and validation are required. Evidence obtained from this study strongly implies that exposure to TNT has altered many biological processes, and that the affected pathways are related to neurological function, oxygen transport, antioxidant response, muscle contraction, calcium binding and signaling, and immune function. Our findings provide new insights into the toxicological mechanisms of TNT at the global gene expression level. This information not only is important to understand how TNT causes toxic effects in a soil organism but also permits comparison with higher organisms where similar biological responses can be expected to further establish a general mechanism of toxic action. Our findings also highlight the many biochemical/physiological aspects that remain to be proven for *E. fetida*, as well as the opportunities to define them.

Acknowledgments

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Supporting Information Available

Supplemental Methods: Detailed description of methods used in the study. Figure S1: A balanced loop hybridization scheme for four treatments with five independent biological replicates. Figure S2A,B: Selection of reference genes for RT-QPCR normalization. Table S1: Array hybridization scheme and Alien RNA spike-in mix. Table S2: Primers designed for RT-QPCR and target cDNA clones. Table S3: Annotation and relative expression of 311 significantly changed cDNAs in the earthworm *Eisenia fetida* exposed to 2,4,6-trinitrotoluene. Table S4: Microarray and RT-QPCR data with 97.5% upper and lower confidence boundaries and standard errors. This material is available free of charge via the internet at http://pubs.acs.org.

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