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Evaluation of tissue and cellular biomarkers to assess 2,4,6-trinitrotoluene (TNT) exposure in earthworms: effects-based assessment in laboratory studies using *Eisenia andrei*

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The lysosomal neutral red retention time (NRRT) assay, a biomarker for lysosomal membrane stability, and the total immune activity (TIA) assay, a measure of non-specific immune system activity, were used in laboratory studies to assess the toxic effects of 2,4,6-trinitrotoluene (TNT) on earthworms (*Eisenia andrei*) *in vivo*. The results were compared with the concentration of TNT and its metabolites in earthworm tissue, as well as standard sublethal toxicity endpoints including growth (i.e. weight change) and reproduction effects from previously published studies. Filter paper experiments indicated a significant decrease in NRRT at $\geq$1.8 µg TNT cm^{-2}, whereas sublethal (weight loss) and lethal effects to earthworms were detected at $\geq$3.5 and 7.1 µg TNT cm^{-2}, respectively. Experiments in artificial soil showed that NRRT effects could be detected at lower TNT concentrations ($\geq$55 mg TNT kg^{-1} soil dry weight) compared with other sublethal endpoints (effects on growth and reproduction). The TIA biomarker did not significantly respond to TNT. Copper (as $CuSO_4$, filter paper contact tests) and 2-chloroacetamide (soil tests), which were used as reference toxicants, also decreased the NRRT. The use of the NRRT assay linked with tissue concentrations of TNT metabolites in earthworms was identified as a potentially appropriate biomarker approach for TNT exposure assessment under laboratory conditions and a novel tool for effects-based risk assessment.

Keywords: explosives, contaminated soil, TNT, energetic substance, polynitroaromatic compound, biomarker.

Introduction

Environmental risk assessment based on the chemical concentrations in soil and water has several limitations (Robidoux *et al.* 2002). The determination of chemical residues in environmental matrices may require an extensive extraction

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procedure, and chemical analysis can be expensive for certain contaminants such as nitro-organic compounds. Moreover, the bioavailability of a chemical cannot be accurately quantified without considering biological effects and tissue residues, for which the analyses can be labour intensive and expensive. Full scale studies of biological effects are often more difficult to carry out and more expensive than chemical analyses of environmental matrices. The use of biomarkers may well prove to be a simpler way of providing realistic and relevant data at the biological level. Adams *et al*. (2001) have suggested that biomarkers should be increasingly used in contaminant risk management.

The biological response of an organism to xenobiotics following absorption and distribution starts with toxicant-induced changes at the cellular and biochemical levels, leading to changes in the structure and function of the organism, and can perhaps ultimately affect the integrity of an ecosystem. Biomarkers can be used to measure these initial changes (Weeks and Svendsen 1996). Moreover, the biomarker-based approach may avoid many of the problems encountered with chemical monitoring by providing direct measurements of toxicant-induced effects on ecological receptors. Earthworms have commonly been considered as useful sentinel organisms and have been used as a bioindicator species for environmental contaminants such as heavy metals (Spurgeon *et al*. 2000).

Svendsen *et al*. (1996) used an earthworm lysosomal biomarker to assess the ecological effects of exposure to metal pollution at an industrial plastics factory. Their results showed that the neutral red retention time (NRRT), the duration for which lysosomes in earthworms coelomocytes could retain a chemical dye (neutral red), was negatively correlated with the concentrations of heavy metal residues in the earthworm (*Lumbricus castaneus*). The NRRT assay may be considered as a non-specific quantitative bioassay that uses lysosomal membrane integrity as a marker for cellular stress (Svendsen and Weeks 1997a). This assay was originally developed to screen for cell culture viability in mammalian toxicity tests, and has now been shown to provide reliable and dose-related responses in certain marine (Lowe *et al*. 1992), freshwater (Svendsen and Weeks 1995) and terrestrial species (Svendsen *et al*. 1996, Svendsen and Weeks 1997a) exposed to xenobiotics. The advantage of this assay is that it is sensitive to a wide range of pollutants and can integrate the combined effects of a complex pollution matrix (Svendsen and Weeks 1997b).

Impairment of the immune system can lead to increased susceptibility to infection, with potential lethal consequences; therefore immune system assays can be used as sublethal biomarkers to assess the non-specific immunotoxicity of environmental contaminants (Goven *et al*. 1993, 1994). The total immune activity (TIA) assay has previously been used to characterize the effects of polychlorinated biphenyls (PCBs) and many pesticides (Goven *et al*. 1993, Bunn *et al*. 1996). This method consists of isolating coelomocytes from earthworms using invasive puncture (Weeks and Svendsen 1996) or non-invasive extrusion (Eyamble *et al*. 1991), and determining the number of cells that show an immune response after a 24 h incubation with rabbit erythrocytes (i.e. single layer rosettes of erythrocytes on the coelomocyte surface, multilayer secretory rosettes and phagocytosis – see Eyamble *et al*. 1991).

Lethality and reproduction tests in the laboratory using the earthworm (*Eisenia andrei*) have proven useful in the assessment of the sublethal effects of explosives such as 2,4,6-trinitrotoluene (TNT) in artificial soil (Robidoux *et al*. 1999, 2000).

However, these methods require a long exposure period (2–8 weeks) and, moreover, the cost and quality of the human resources needed to complete these tests are relatively high. Alternatively, earthworm contact tests (using pure substances or soil extracts presented on filter paper) can rapidly and inexpensively screen for chemicals that are potentially toxic (lethal) to earthworms. This latter test is designed to provide contact between the worm and the chemical as close as would be found in soil (Neuhauser *et al*. 1986), but exposure doses are generally difficult to relate to soil situations due to differences in bioavailability.

The NRRT assay appears to be a sensitive earthworm (*Lumbricus* and *Eisenia* species) biomarker for soils contaminated by heavy metals such as copper, cadmium, lead, antimony (Weeks and Svendsen 1996, Svendsen *et al*. 1996, Svendsen and Weeks 1997a,b) and nickel (Scott-Fordsmand *et al*. 1998). Although some work has been done on benzo[*a*]pyrene and the organophosphate pesticide chloropyrifos (Eason *et al*. 1999, Booth *et al*. 2001), little is known about the potential of this biomarker for other organic contaminants, and there is no previously published data for explosive compounds. In addition, earlier studies have suggested that residual acetonitrile (remaining following the solvent-evaporation step of the soil-spiking procedure) may be toxic to *E. andrei* (Robidoux *et al*. 2000, 2001). The present study compares the NRRT and TIA assay responses with other published endpoints of toxicity (lethality, growth or weight loss, and fecundity) using earthworms (*E. andrei*) exposed to TNT, acetonitrile (the solvent vehicle for TNT) and two reference toxicants ($CuSO_4$ and 2-chloroacetamide).

Materials and methods

Chemicals and reagents

TNT was obtained from ICI Explosives Canada (McMasterville, Quebec, Canada). Neutral red dye, rabbit red blood cells, Sigmacoat and Eosin Y were obtained from Sigma Chemical (St Louis, Missouri, USA). Other chemicals such as the reference toxicants ($CuSO_4$ and 2-chloroacetamide) were American Chemical Society (ACS) reagent grade and were obtained from Aldrich (Milwaukee, Wisconsin, USA). Deionized water (ASTM, type II) was obtained from a Super-Q water purification system (Millipore) and Zenopure Mega-90, and was used throughout the study. Glassware was washed with phosphate-free detergent followed by rinses with acetone, nitric acid 10% (v/v) and finally with deionized water.

Artificial soil consisting of 70% (w/w) grade 4010 silica sand (Unimin Canada, Canada), 20% colloidal kaolinite clay (CAS: 1332-56-7) and 10% 2 mm sieved Canadian sphagnum peat was prepared according to the Organization for Economic Cooperation and Development (OECD) method (OECD 1984). Peat, sand and clay were obtained from local suppliers. Calcium carbonate (1% w/w) was used to adjust the pH of the wetted substrate to 6.0 ± 0.5.

Balanced salt solution (BSS) (Goven *et al*. 1994) was freshly prepared in demineralized water (71.5 mM NaCl, 4.8 mM KCl, 3.8 mM $CaCl_2$, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4 and 4.2 mM $NaHCO_3$). The solution was then adjusted to pH 7.3 with NaOH 0.1 N and filtered through a 0.22 µm membrane.

Earthworms

The oligochaetes *E. andrei*, or *E. fetida andrei* were obtained from Carolina Biological Supply (Burlington, North Carolina, USA) and were initially used to establish the laboratory cultures. Animals were maintained in earthworm bedding (Magic Products, Amherst Jct, Wisconsin, USA) supplemented with dry cereal (Magic Worm Food, Magic Products) at $20 \pm 1°C$, 70–80% humidity and a light/dark cycle of 16 h on/8 h off. Only adult earthworms (ranging from 300 to 600 mg wet weight) having a well-developed clitellum were used in the toxicity tests.

Exposure to toxicants on filter paper

The filter paper contact test permits the determination of the relative acute toxicity of chemicals (Neuhauser *et al*. 1986, OECD 1984). The earthworms *E. andrei* were exposed to chemicals dissolved in

aqueous solution or in a volatile organic solvent (acetonitrile) according to the OECD guidelines as described in Robidoux *et al*. (1999). The effects of TNT and $CuSO_4$ on the NRRT and TIA responses in *E. andrei* were assessed in 10 worms (each one placed in a different vial) exposed to each test concentration and to controls (negative control and solvent vehicle control). Each clean glass vial replicate was lined with a 60 cm^2 filter paper (Whatman no. 1). A fixed volume (1 ml) of solvent vehicle containing the test compounds in a range of concentrations was applied to each filter paper. For TNT, acetonitrile was used as the solvent vehicle in order to provide high concentrations of TNT (32 to 849 mg l^{-1}). In the $CuSO_4$ studies, deionized water was used as the vehicle. To provide an even distribution of the test compounds in the filter paper, the solvent vehicle was evaporated gently under a jet of air and then the filter paper was rehydrated using 1 ml of water. For each test concentration, the number of surviving earthworms was recorded after 48 and 72 h of exposure by testing their reaction to a mechanical stimulus at the anterior end. The wet mass of each worm was recorded at the start of the test and after 72 h of exposure. The NRRT and TIA were determined for three or more survivors per concentration unless otherwise stated.

Exposure to toxicant in spiked soil

The earthworm biomarkers were also examined under the conditions used for the *E. andrei* soil toxicity test (ISO 1996) as described in Robidoux *et al*. (2000, 2001). For the present study, experiments were carried out in OECD artificial soil spiked with toxicant. Ten earthworms per replicate jar were exposed, and four replicates per concentration were used, unless otherwise stated. The biomarker measurements (NRRT and TIA) were carried out using at least three survivors per replicate jar after 28 days of exposure. These effects were compared with published reports describing the lethality and growth inhibition in adult earthworms determined after 28 days, and reproduction parameters (inhibition of cocoon production and hatching, survival and growth of juveniles) after 56 days of exposure to toxicant (Robidoux *et al*. 2000).

The present study also examined the effect of residual acetonitrile on NRRT and TIA measurements after 28 days of exposure to the residual amount of solvent. In brief, 500 g of dry soil was spiked separately with three different volumes of acetonitrile to produce three nominal concentrations (20, 40 and 200 µl g^{-1}). The soil was then mixed daily and allowed to evaporate for varying periods (3, 4, 15 and 30 days) under a chemical hood, as described by Robidoux *et al*. (2000, 2001). Experiments were carried out using negative (water) controls and soils spiked with acetonitrile. Thus, acetonitrile was added to soils that were then left to evaporate for 3 days (20 µl g^{-1} dry soil) or 4 days (20 and 40 µl g^{-1} dry soil) under the chemical hood. Soil containing 200 µl g^{-1} acetonitrile was allowed to evaporate in the chemical hood until the acetonitrile concentration in the soil reached <1.0 µl g^{-1} dry soil (Robidoux *et al*. 2000). The concentration of residual acetonitrile was measured over the 15 and 30 day evaporation periods using gas chromatographic analysis as described below.

Assays using TNT as the contaminant (55–881 mg kg^{-1}) and 2-chloroacetamide (2–35 mg kg^{-1}) as the reference toxicant were carried out in quadruplicate at five or six different sublethal concentrations as described previously by Robidoux *et al*. (2000). Dry artificial soil was prepared and 500 g (dry weight) portions were placed in clean 1 litre Mason-type glass jars for each replicate. The test substances were first dissolved in their respective solvents: acetonitrile for TNT (due to its limited aqueous solubility) and water for 2-chloroacetamide. A range of defined concentrations were used to ensure that all soils could be spiked using equal amounts of solvent vehicle (10 ml of acetonitrile per 500 g of dry soil for TNT). TNT (in acetonitrile) was added and mixed into the soil of each replicate, and the solvent was allowed to evaporate for 4 days in a chemical hood in order to obtain an acetonitrile concentration of <1.0 µl g^{-1} dry soil. A solvent vehicle control (acetonitrile only, no TNT added) was also used. At the beginning of the experiments, the soil (500 g dry weight) was rehydrated to 75% of its water-holding capacity. Earthworms were acclimated in clean OECD soil for 1–3 days prior to inoculation. Ten *E. andrei* were individually washed with deionized water and gently dried on paper, weighed and added to each replicate jar containing the test soil sample. Glass jars were closed using a geotextile and lids with 1.6 mm air holes. Dry cereal (2 g dry weight) was used to feed the earthworms and was added at the beginning of the experiment and then once weekly. The pH values were measured before and after each test using a 1:5 (v/v) suspension of soil in water (ISO 1994).

NRRT assay

The determination of the NRRT was done after exposure to the toxicant (after 3 days of exposure for the filter paper contact test and after 28 days of exposure for the soil test) using the histochemical staining technique of Weeks and Svendsen (1996). A neutral red stock solution (20 mg neutral red dissolved in 1 ml of dimethyl sulphoxide) was freshly prepared. Subsequently, 10 µl of the stock solution was diluted in 2.5 ml of BSS, giving a neutral red working concentration of 80 µg ml^{-1}. The neutral red working solution was renewed each hour to avoid crystallization of the non-polar neutral red in the aqueous BSS solution. A small sample of coelomic fluid (about 50 µl) was taken from each selected live worm by carefully inserting a detachable 24 gauge needle (5/8 inch; Becton Dickinson, New Jersey, USA) on a 1 ml tuberculin syringe (0.01 ml increments; Becton Dickinson) containing approximately

50 µl of BSS directly into the coelomic cavity in the region posterior to the clitellum. A 25 µl aliquot of this mixture was placed onto a microscopic slide, and the cells were allowed to adhere to the slide for 60 s prior to the application of 25 µl neutral red working solution (80 µg ml^{-1}) and a coverslip. Each slide was scanned continuously for 1 min at 3–6 min intervals under a light microscope (400×). The observation endpoint was taken when the ratio of coelomocytes with fully stained cytoplasm reached >50% of the total number of coelomocytes counted. This interval (in minutes) was recorded as the NRRT.

TIA assay

In order to assess the potential of the earthworm immune response to TNT, another sublethal biomarker, the TIA was tested using immunological approaches described by others (Goven *et al*. 1993, Bunn *et al*. 1996). For this procedure, rabbit red blood cells were reconstituted with 10 ml of BSS before being left overnight to rehydrate prior to use.

A quantity (confirmed by weight) of coelomic fluid/BSS mixture (taken from each live worm selected for the NRRT assay) was incubated in Eppendorf tubes (previously coated with 'Sigmacoat' to minimize adhesion of coelomocytes to the tubes) with a proportional volume (1 µg:1 µl) of reconstituted rabbit erythrocytes in BSS (rabbit red blood cells diluted 300 times) for 24 h at 20°C. Thereafter, 25–40 µl of the mixture was taken from the tubes, placed on a microscopic slide and stained with 4 µl Eosin Y solution (2 mg Eosin Y in 1 ml of BSS) prior to adding a coverslip. At least 100 coelomocytes were then examined under a light microscope (400×), and the proportion of live (coloured green) coelomocytes showing immune activity (phagocytosis of the erythrocytes, erythrocytes rosettes or secretory rosettes) were assessed. The immune activity in earthworms exposed to contaminants was compared with that in the control earthworms.

TNT and its metabolites in earthworm tissues after exposure in soil

After 28 days of exposure in soil, five (out of the 10 per replicate) earthworms that had been exposed to different TNT concentrations (see above) were frozen at −85°C for later chemical analysis. TNT and its metabolites were extracted from the earthworm tissue using the acetonitrile technique as described by Renoux *et al*. (2000).

Chemical analyses

Concentrations of TNT in acetonitrile solution were measured by high performance liquid chromatography (HPLC) using the US-EPA (1997) SW-846 Method 8330 as described previously (Robidoux *et al*. 2000). Residual acetonitrile was determined as described in an earlier work (Robidoux *et al*. 2000) utilizing a Perkin Elmer Sigma 2000 Gas Chromatograph equipped with a flame ionization detector (FID) using helium as the carrier gas. The limit of detection was 100 p.p.b., and the precision was >95%. The water content of the soil was obtained by drying soil at 103 ± 2°C for 16 h. All toxicant concentrations in soil are expressed on a dry weight basis.

Quality control

All experiments were done using 10 (contact test) or four (soil test) replicates, and included negative controls (no toxic substances added) and solvent controls. As mentioned above, $CuSO_4$ (contact test, Svendsen and Weeks, 1997a) and 2-chloroacetamide (soil test, Gibbs *et al*. 1996, Gunderson *et al*. 1997) were chosen as reference toxicants. Mortality, growth and reproduction endpoints in the reference toxicants and other established criteria (e.g. response in the negative control, temperature) were verified against laboratory in-house control data to validate the toxicological analyses.

Data analysis

Toxicity endpoints such as the lowest observable effect concentration (LOEC) and no observed effect concentration (NOEC) were estimated using the ToxCalc program (version 5.0, Tidepool Scientific Software, McKinleyville, California, USA). Statistical methods for point estimates included maximum likelihood regression, Spearman–Karber methods and linear interpolation with bootstrapping. Dunnett's multiple comparison test was used to determine the LOEC and NOEC. Data was expressed as the mean ± standard deviation (SD) or standard error (SE). SD was used for the NRRT response to toxicant on filter paper as well as for survival. SE was used for NRRT (four replicates) and growth (three to four replicates) responses to toxicant exposure in soil.

The NRRT data were analysed with a *t*-test for independent samples (if normal distribution and equal variance were confirmed) or with a non-parametric (Mann–Whitney U test or Wilcoxon matched pairs test) to observe differences ($p \leq 0.05$) among the treatment concentrations.

Results

NRRT response to toxicants on filter paper

Exposure to TNT on filter paper significantly decreased earthworm NRRT at concentrations $>1.8\,\mu g\,cm^{-2}$ ($p < 0.05$) (NOEC $= 0.9\,\mu g\,cm^{-2}$). This response to TNT was more sensitive than survival or changes in body weight when carried out under the same conditions, considering results from this and earlier studies (Robidoux *et al.* 1999). Figure 1 shows the results of a representative filter paper contact test experiment, and compares the NRRT measurements to survival and body weight changes as responses to TNT.

The NRRT in earthworms exposed to $CuSO_4$ was also significantly reduced compared with controls at concentrations $\geq 0.208\,\mu g\,Cu\,cm^{-2}$ ($p < 0.05$) (NOEC $= 0.104\,\mu g\,cm^{-2}$), except for $0.417\,\mu g\,Cu\,cm^{-2}$ ($p = 0.06$). Figure 2 shows the results of a filter paper contact test experiment, and compares the NRRT to survival and body weight changes. Significant earthworm lethality was observed with $CuSO_4$ exposures $\geq 0.833\,\mu g\,Cu\,cm^{-2}$, whereas a significant weight loss was observed at $\geq 0.208\,\mu g\,cm^{-2}$.

Survival and growth of adult earthworms in response to acetonitrile in soil

The survival and growth of earthworms in experiments using different acetonitrile spiked-soil concentrations and evaporation periods (tables 1 and 2; Robidoux *et al.* 2000, 2001) were not significantly different to those of control groups.

NRRT response to toxicants in soil

Acetonitrile-spiked soils may affect the earthworm NRRT depending on the concentration and evaporation time used. A similar NRRT (59.8 ± 0.6 min,

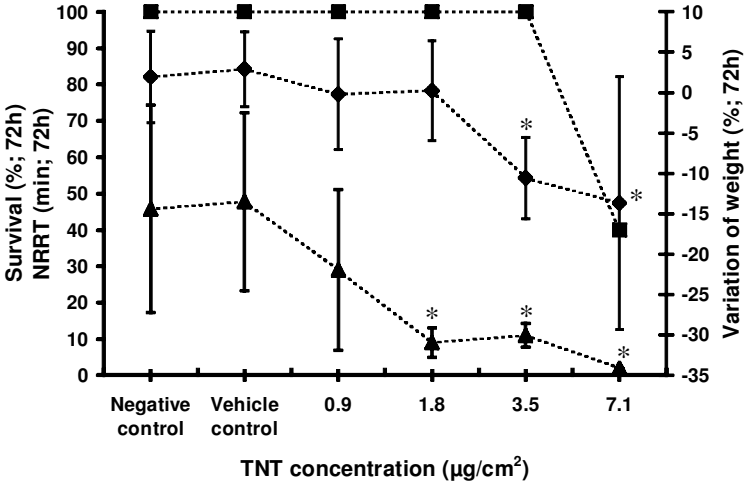

Figure 1. Lethal and sublethal effects of TNT in the oligochaete *E. andrei* using the filter paper contact test method. ■, survival ($n = 10$ earthworms per concentration at start of experiment); ◆, weight loss ($n = 10$, except for the higher concentration [$n = 4$]); ▲, NRRT ($n = 4$, except for the higher concentration [$n = 2$]). Error bars, ±SD. * indicates a significant difference ($p \leq 0.05$) compared with negative and vehicle controls.

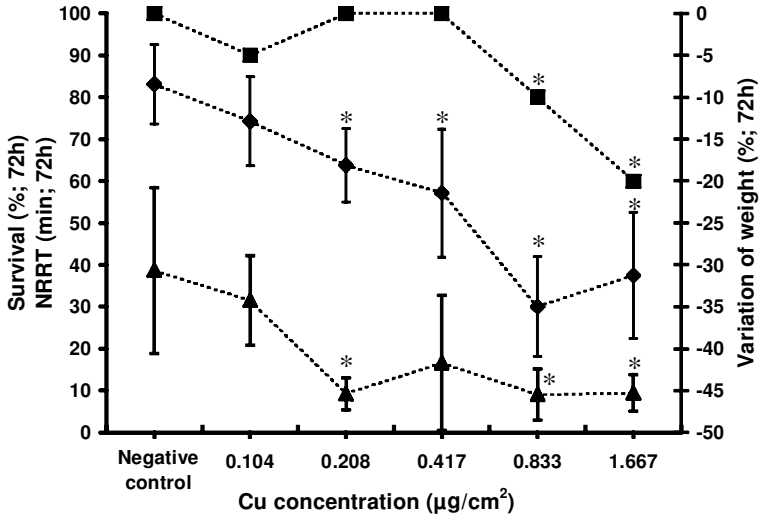

Figure 2. Lethal and sublethal effects of copper ($CuSO_4$) in the oligochaete *E. andrei* using the filter paper contact test method. ■, survival ($n = 10$ earthworms per concentration at start of experiment); ◆, weight loss ($n = 6$–10); ▲, NRRT ($n = 3$–5). Error bars, ±SD. * indicates a significant difference ($p \leq 0.05$) compared with negative and vehicle controls.

experiment 1, table 1) was observed in earthworms from the 40 µl g^{-1} spiked soil as in controls (51.5 ± 2.1 min, experiment 1, table 1) when using a 3 day solvent evaporation period. Acetonitrile-spiked soils (20 and 40 µl g^{-1}) were also not toxic after a 4 day solvent evaporation period, giving a high NRRT (59.5 ± 0.9 min and 52.0 ± 3.7 min, respectively, experiment 2, table 1). Using a high initial soil concentration of acetonitrile (200 µl g^{-1}) and a longer evaporation period (15 days), the residual acetonitrile concentration was 0.90 ± 0.26 µl g^{-1} dry soil. The NRRT was relatively low (34.7 ± 5.8 min, experiment 3, table 2) but was similar to the control group (46.3 ± 3.0 min) for that experiment. Using a longer evaporation period (30 days) and a high initial soil concentration of acetonitrile (200 µl g^{-1}), the residual acetonitrile concentration was 0.07 ± 0.03 µl g^{-1} dry soil. NRRT assays conducted on the adults showed results (39.8 ± 1.4 min, experiment 4, table 2) similar to those after a 15 day evaporation period. The NRRT was significantly different compared with the control groups (50.7 ± 4.3 min, experiment 4, table 2), but was not different from that of the controls in other experiments (experiments 1, 2 and 3, tables 1 and 2).

Experiments showed an inverse relationship between TNT concentration and the NRRT of coelomocytes extruded from exposed earthworms (figure 3). The NRRT was significantly decreased compared with controls at TNT concentrations ≥55 mg kg^{-1}. Adult earthworms exposed for 28 days to the reference toxicant 2-chloroacetamide also showed a significant decrease in NRRT (figure 4) compared with the negative control group. The NRRTs at 10 and 15 mg kg^{-1} of 2-chloroacetamide were significantly different from controls. However, the highest concentration tested with survivors (25 mg kg^{-1}) gave NRRT values that were not significantly different from the control group; in this case adult survival was low (40%) compared with the 100% survival seen at lower concentrations (data not shown). No survivors were found at the highest 2-chloroacetamide concentration used in this study (35 mg kg^{-1} dry soil).

Table 1. Responses of earthworm (*E. andrei*) to acetonitrile-evaporated soil (low nominal concentrations).

	Nominal acetonitrile soil concentrations				
	3 days of solvent evaporation (experiment 1)		4 days of solvent evaporation (experiment 2)		
Parameter	Negative control 1	20 µl g^{-1} dry soil	Negative control 2	20 µl g^{-1} dry soil	40 µl g^{-1} dry soil
Adult survival/replicate at 28 days	100%[b]	100%[b]	100%[b]	100%[b]	100%[b]
Adult growth/worm[a] at 28 days (mean ± SE)					
Change in weight	105.0 ± 24.4 mg[b]	69.4 ± 27.8 mg[b]	104.0 ± 22.9 mg[b]	137.8 ± 28.4 mg[b]	177.8 ± 33.5 mg[b]
% change	23.0 ± 4.8%	16.0 ± 6.2%	22.4 ± 6.0%	28.8 ± 7.2% (NS)	39.2 ± 9.5% (NS)
NRRT (mean ± SE)	51.5 ± 2.1 min	59.8 ± 0.6 min (NS)	52.0 ± 3.7 min	59.5 ± 0.9 min (NS)	52.0 ± 4.6 min (NS)

n = replicates per concentration. Negative control: rehydrated (75% water-holding capacity) artificial soil, no contaminant added. Acetonitrile concentrations: concentration of acetonitrile added and evaporated prior to rehydrating (75% water-holding capacity) artificial soil. Adult survival and growth data were partially taken from Robidoux *et al.* (2000).

[a] Mean change in body weight during the experiment (average of initial minus final body weight).

[b] Published earlier (Robidoux *et al.* 2000).

NS, not significantly different ($p > 0.05$) compared with negative control group.

Table 2. Responses of earthworm (*E. andrei*) to acetonitrile-evaporated soil (high nominal concentrations).

	Nominal acetonitrile soil concentrations			
	15 days of solvent evaporation (experiment 3)		30 days of solvent evaporation (experiment 4)	
Parameter	Negative control 3	200 µl g^{-1} dry soil	Negative control 4	200 µl g^{-1} dry soil
Adult survival/replicate at 28 days (mean ± SD)	97.5 ± 5.0%[b]	100%[b]	100%[c]	100%[c]
Adult growth/worm[a] at 28 days (mean ± SE)				
Change in weight	48.0 ± 8.7 mg[b]	89.4 ± 22.7 mg[b]	104.4 ± 12.8 mg[c]	153.3 ± 19.5 mg[c]
% change	9.8 ± 1.9%	18.1 ± 4.7% (NS)	37.5 ± 4.7%	44.9 ± 5.1% (NS)
NRRT (mean ± SE)	46.3 ± 3.0 min	34.7 ± 5.8 min (NS)	50.7 ± 4.3 min	39.8 ± 1.4 min[d]

$n = 3$ (15 day experiment) or 4 replicates (30 day experiment) per concentration. Negative control: rehydrated (75% water-holding capacity) artificial soil, no contaminant added. Acetonitrile concentrations: concentration of acetonitrile added and evaporated prior to rehydrating (75% water-holding capacity) artificial soil. Adult survival and growth data were taken from Robidoux *et al.* (2000, 2001).

[a] Mean change in body weight during the experiment (average of initial minus final body weight).

[b] Published earlier (Robidoux *et al.* 2000).

[c] Published earlier (Robidoux *et al.* 2001).

[d] Significant difference ($p \leq 0.05$) compared with control groups (*t*-test).

NS, not significantly different ($p > 0.05$) compared with negative control group.

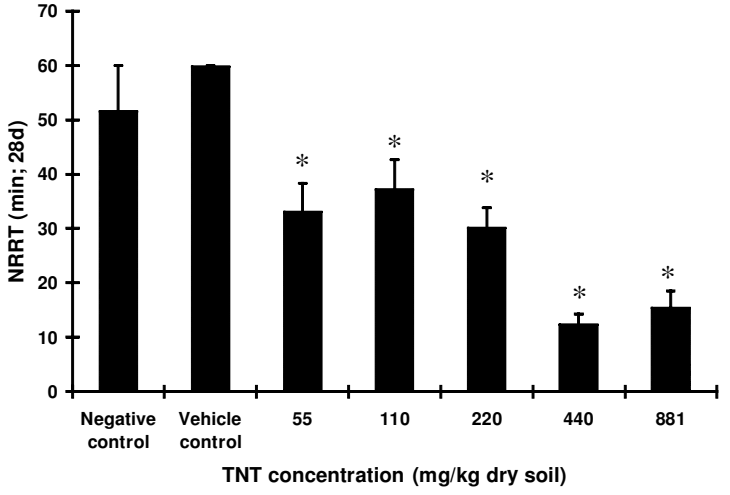

Figure 3. NRRT in earthworms (*E. andrei*) exposed to increasing nominal concentrations of TNT in artificial soil for 28 days under laboratory conditions ($n = 4$–10 worms/replicate, four replicates per concentration, 19–25 worms per concentration). The negative control consisted of rehydrated (75% water-holding capacity) artificial soil with no contaminant added. The solvent control consisted of 10 ml of acetonitrile added and evaporated prior to rehydration (to 75% water-holding capacity) of the artificial soil. Error bars, ±SE. * indicates a significant difference ($p \leq 0.05$) compared with negative and vehicle controls.

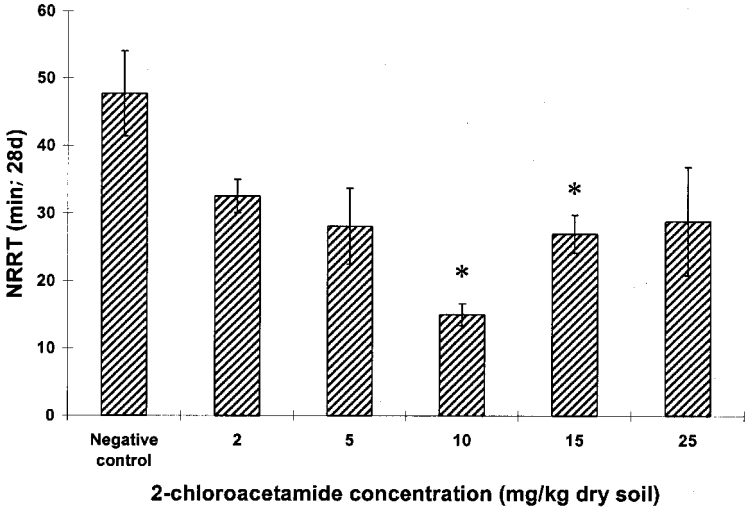

Figure 4. NRRT in earthworms (*E. andrei*) exposed to increasing nominal concentrations of 2-chloroacetamide in artificial soil for 28 days under laboratory conditions ($n = 4$–10 worms/replicate, four replicates per concentration, 10–17 worms per concentration). The negative control consisted of rehydrated (75% water-holding capacity) artificial soil with no contaminant added. Error bars, ±SE. * indicates a significant difference ($p \leq 0.05$) compared with controls. No survivors were found at an 2-chloroacetamide concentration of 35 mg kg^{-1} dry soil.

TIA biomarker

Preliminary studies using the TIA assay showed no significant responses in earthworms exposed to TNT on filter paper (0.9–7.1 µg cm^{-2}) or in soil (55–881 mg kg^{-1}; data not shown).

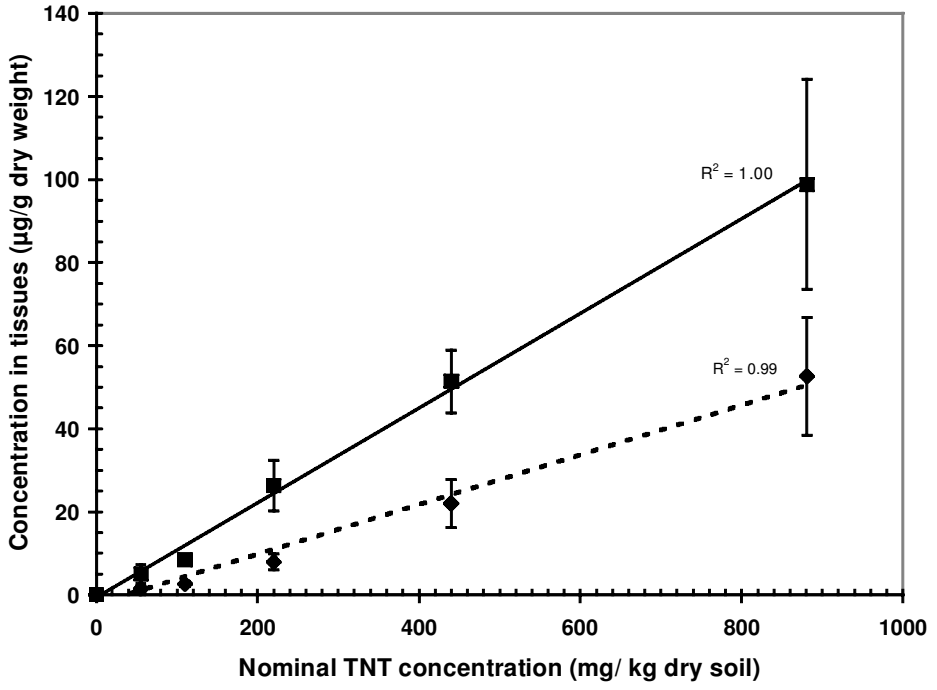

Figure 5. Tissue concentration of TNT metabolites in earthworms ($\mu g\,g^{-1}$) exposed for 28 days to different nominal concentrations of TNT in artificial soil. Metabolites in earthworm tissues included 2-ADNT (■, $n = 4$ per concentration) and 4-ADNT (◆, $n = 4$ per concentration). The equations of regression were $y = 0.11x - 0.60$ for 2-ADNT (solid line) and $y = 0.06x - 2.23$ for 4-ADNT (broken line).

TNT metabolites in earthworm tissue

Using linear regression, a significant correlation was found between the mean tissue concentrations of TNT metabolites in earthworms and the nominal TNT concentration in TNT-spiked artificial soil ($R^2 = 0.99$ and 1.00 for 2-amino-4,6-dinitrotoluene [2-ADNT] and 4-amino-2,6-dinitrotoluene [4-ADNT], respectively; figure 5). TNT was not detected in earthworms after 28 days of exposure to the TNT-spiked artificial soil samples, consistent with earlier results using 14 day exposure to TNT-spiked forest soil (Renoux *et al.* 2000).

NRRT versus concentrations of TNT and its metabolites

Analysis of the mean NRRT in earthworm coelomocytes and the nominal TNT concentrations in artificial TNT-spiked soil indicated a significant linear correlation ($R^2 = 0.82$; data not shown). However, the NRRT was not significantly correlated with the individual metabolites in earthworm tissues ($R^2 = 0.33$ and 0.55 for 2-ADNT and 4-ADNT, respectively; data not shown).

Discussion

This study examined two biomarker responses (NRRT and TIA) to TNT exposure and compared these responses with previous toxicity data (Robidoux

et al. 1999, 2000). The results indicated that NRRT is a potential biomarker of exposure to TNT. The NRRT assay has been previously identified as an appropriate biomarker for a range of metals (Weeks and Svendsen 1996, Svendsen and Weeks 1997a,b, Scott-Fordsmand *et al.* 1998). However, as noted earlier (Scott-Fordsmand *et al.* 1998), NRRT is a sensitive endpoint and shows a large individual variation amongst the earthworms exposed to a particular concentration. TNT did not significantly affect the immune response measured by the TIA assay using the 72 h and 28 day exposure conditions described above.

Previous experiments have shown that TNT was lethal to the earthworm *E. andrei* using the filter paper contact test, with the LOEC from different experiments ranging from >2.78 to $14.15\,\mu g\,cm^{-2}$ (Robidoux *et al.* 1999). Using the filter paper test, the NRRTs were lower at lethal or sublethal concentrations of TNT (3.5 and $7.1\,\mu g\,cm^{-2}$; figure 1) than in controls. In addition, the NRRTs were also lower at a TNT concentration that had no significant lethal or weight loss effects ($1.8\,\mu g\,cm^{-2}$). A TNT concentration–response relationship was observed. Experiments with $CuSO_4$, used as a reference toxicant, also indicated that NRRT is a sensitive endpoint, showing effects at sublethal concentrations (figure 2). Variability in NRRT between experiments appeared to be relatively high (45.8 ± 28.5 min versus 38.7 ± 19.8 min in the negative controls in the TNT and $CuSO_4$ experiments, respectively) and was probably due to biological variability, the selected organisms and the test conditions (exposure on filter paper is stressful to earthworms).

Residual sublethal toxicity (as measured by adult growth after 28 days of exposure or reproduction after 56 days of exposure) of acetonitrile in soil after evaporation under a chemical hood has recently been reported (Robidoux *et al.* 2000, 2001). The results, based on headspace gas chromatographic FID analyses of residual acetonitrile, indicated that the concentration of acetonitrile fell to $\leq 1\,\mu l\,g^{-1}$ after 2 days in the $40\,\mu l\,g^{-1}$ dry weight spiked soil or after 15 days using the higher initial concentration ($200\,\mu l\,g^{-1}$ dry weight). Soils spiked with 20 and $40\,\mu l\,g^{-1}$ acetonitrile were not toxic after a 3 or a 4 day solvent evaporation period and NRRTs were relatively high (59.8 ± 0.6 and 59.5 ± 0.9 min for $20\,\mu l\,g^{-1}$ in experiments 1 and 2, and 52.0 ± 4.6 min for $40\,\mu l\,g^{-1}$ in experiment 2, table 1). Using a high initial soil concentration of acetonitrile ($200\,\mu l\,g^{-1}$) and a longer evaporation period (15 days), a significant decrease in reproduction of *E. andrei* was observed (Robidoux *et al.* 2000), but adult survival and growth (experiment 3, table 2) were not affected. The NRRT was relatively low (34.7 ± 5.8 min, experiment 2, table 2) but was not significantly lower than the control groups. Thus, after the 15 day evaporation period, the acetonitrile soil concentration ($0.90 \pm 0.26\,\mu l\,g^{-1}$ dry soil; Robidoux *et al.* 2000) was decreased by 99.6%, but residual sublethal toxicity of acetonitrile shown earlier (Robidoux *et al.* 2000) was not detected by this NRRT assay.

A longer evaporation period (30 days) that permitted the decrease of the high initial acetonitrile soil concentration of $200\,\mu l\,g^{-1}$ to $0.1\,\mu l\,g^{-1}$ was not associated with a decrease in the reproduction of *E. andrei* or in adult growth (Robidoux *et al.* 2001). NRRT assays conducted on the adults exposed to this acetonitrile-spiked soil also showed results (39.8 ± 1.4 min, experiment 4, table 2) similar to those measured in experiments with shorter evaporation times (experiments 1, 2 and 3, tables 1 and 2). However, the NRRT was significantly lower than that of the control group. This means that NRRT may be sensitive to low concentrations of

acetonitrile ($0.1\,\mu g\,g^{-1}$) that are not detected using the earthworm reproduction test, as shown earlier (Robidoux *et al.* 2001). However, the data indicates that the sensitivity of the NRRT assay may depend on responses in control groups, especially when NRRT values ranged from 30 to 60 min. In addition, a significant response with the NRRT biomarker, which also depends on species and exposure conditions, is often lower than 25 min (Svendsen *et al.* 1996, Svendsen and Weeks 1997a, Scott-Fordsmand *et al.* 1998, Spurgeon *et al.* 2000, Booth *et al.* 2001). This suggests that a low NRRT value (<25 min) indicates the presence of a contaminant (such as residual organic solvent) or other sources of stress (environmental conditions).

Recent experiments (Robidoux *et al.* 2000) have indicated that TNT has significant effects on *E. andrei* growth and reproduction (LOEC = 110 mg TNT kg^{-1} dry soil). Production of cocoons and juveniles were reduced by exposure to $\geq 220\,mg\,kg^{-1}$ of TNT, whereas the total biomass of juveniles was decreased at $\geq 110\,mg\,kg^{-1}$ of TNT. Experiments in OECD artificial soil also showed effects on the NRRT at sublethal concentrations (figure 3). Earthworms exposed to TNT had lower NRRTs compared with controls for all concentrations tested (55–881 mg TNT kg^{-1} dry weight), whereas other effects were detected at higher concentrations (growth: $881\,mg\,kg^{-1}$; reproduction: $110\,mg\,kg^{-1}$) (Robidoux *et al.* 2000). Thus, the levels of stress on earthworms (reflected in the NRRT using coelomocytes) is significant at low concentrations of TNT (those not causing measurable reproduction effects).

The strong relationship between the mean NRRT in earthworm coelomocytes and nominal TNT concentrations in artificial TNT-spiked soil suggests that the NRRT may be used as a biomarker of effect for TNT-contaminated soils. However, nothing is known about interference with other chemicals (such as TNT-associated substances, metals) also found in soils contaminated with explosives.

Similar observations were seen for the 2-chloroacetamide experiments (figure 4). Significant effects on the NRRT were observed at concentrations as low as $10\,mg\,kg^{-1}$ of 2-chloroacetamide, whereas effects on reproduction were not detected at $\leq 15\,mg\,kg^{-1}$ dry weight (Robidoux *et al.* 2000). However, the NRRT response to the highest concentration used at which survivors were found was not significantly different to that of the negative (water) controls. This could be due to the low survival rate (40%) at this concentration, resulting in only the strongest and least affected individuals being used for the assay.

Several studies of life-cycle parameters (cocoon production, weight change and survival) and NRRT responses have been undertaken in several species (*E. andrei*, *E. fetida*, *E. veneta*, *Lumbricus terrestris*, *L. rubellus*, *Aporrectodea rosea* and *A. caliginosa*), but these have mainly been concerned with metal contamination (Svendsen and Weeks 1997a; Harreus *et al.* 1997; Olesen 1998; Scott-Fordsmand *et al.* 1998; Spurgeon *et al.* 2000). From these studies it was clear that reductions in lysosomal integrity (as measured by the NRRT) generally occurred earlier and at lower doses than the measured life-cycle effects.

Prior to this study nothing was known about the relationship between responses of biomarkers (such as the NRRT) and potential ecological consequences (such as the decrease in earthworm populations) for TNT and other explosives. This study, along with our previous data (Robidoux *et al.* 2000), suggests that exposure to low concentrations of explosive contaminants (such as TNT) that lead

to sublethal toxicity (such as decrease in fecundity) may be detected by the NRRT biomarker. The NRRT assay has a potential use in environmental risk assessment and routine monitoring, as it is likely to provide sufficient and accurate warning of any impending ecological damage at contamination levels below that found to cause physiological damage to earthworms.

The relationship between nominal TNT concentration in TNT-spiked artificial soil and the mean TNT metabolite concentrations in earthworms showed that the concentration of metabolites in earthworm tissues reflect exposure to TNT (and/or its metabolites) under the conditions used. Thus, the present study and other recent work (Renoux *et al.* 2000) suggest that TNT metabolite (and not the parent compound) concentrations in earthworm tissue could be used as a biomarker of short- (24 h) or long-term (28 day) exposure for TNT-contaminated soils.

Conclusions

This study has shown that a biomarker approach using the NRRT assay and tissue concentrations of TNT metabolites in earthworms is potentially appropriate for TNT exposure assessment under laboratory conditions, and could be used as a tool for effects-based risk assessment. Moreover, in this study, the NRRT gave a clear response for all TNT concentrations associated with sublethal effects (changes in body weight, growth and fecundity). In addition, NRRT was more sensitive than conventional sublethal endpoints. Preliminary studies examining TNT metabolite concentrations in tissues showed that this biomarker approach should be developed further for explosives exposure assessment. Further validation is needed before the NRRT can be used as an *in situ* field monitoring tool for exposure and risk assessment.

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