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Coelomocyte biomarkers in the earthworm *Eisenia fetida* exposed to 2,4,6-trinitrotoluene (TNT)

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Abstract Contamination by 2,4,6-trinitrotoluene (TNT) is a global environmental problem at sites of former explosive production, handling, or storage, and could have deleterious consequences for human and ecological health. We investigated its sublethal effects to *Eisenia fetida*, using two nonspecific biomarkers. In coelomocytes of earthworms exposed 24, 48, or 72 h, we evaluated DNA damage (comet assay) and neutral red retention time (NRRT), using the filter paper contact test. Both percentage of damage (D%) and calculated damage index showed significant DNA damage at almost all concentrations, at all time points assayed. Along exposure time, two different patterns were observed. At the lower TNT concentrations (0.25–0.5 μg/cm^2) an increased DNA migration at 48 h, with a decrease close to initial levels after 72 h exposure, was observed. This decrease could be attributed to activation of the DNA repair system. At higher concentrations (1.0–2.0 μg/cm^2), the high DNA damage observed remained constant during the 72 h exposure, suggesting that the rate of DNA repair was not enough to compensate such damage. Analysis of NRRT results showed a significant interaction between time and treatment. After 48 h, a significant decrease was observed at 4.0 μg/cm^2. After 72 h, NRRT presented a concentration-dependent decrease, significantly different with respect to control at 0.5, 1.0, 2.0, and 4.0 μg/cm^2. The two assayed methods, performed on the same sample, showed clear responses to sublethal TNT exposure in *E. fetida*, providing sensitive unspecific biomarkers of cell injury and DNA damage.

Keywords 2,4,6-trinitrotoluene (TNT) · *Eisenia fetida* · Coelomocytes · Comet assay · Neutral red retention time · Biomarkers

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Introduction

Contamination by 2,4,6-trinitrotoluene (TNT), a recalcitrant compound, is a global environmental problem at sites of former explosive production,

handling, or storage. This compound has been included as a priority pollutant due its toxicity and potential carcinogenicity (Casabé et al. 2003; Bolt et al. 2006). Environmental concentrations of TNT and its degradation products as high as 130 mg/l in water and from 2–700,000 mg/kg in sediments have been informed (Sims and Steevens 2008). Conversion of polluted soils for agricultural and urban uses could have deleterious consequences for human and ecological health. In Argentina, soils of an area in which an explosive manufacturing plant has been operating for 50 years were highly contaminated with TNT and in need of remediation. TNT concentrations in sediments of the wastewater disposal lagoon ranged from 7–800 mg/kg (Fuchs et al. 2001).

Currently, there are serious challenges in conducting risk assessments and setting remediation goals in TNT contaminated sites, due to gaps in the knowledge of the environmental fate and effects of this explosive and by-products (Lewis et al. 2004). TNT is readily metabolized at different trophic levels by nitroreduction into monoamine metabolites (2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT)) and then into diamino metabolites (2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene); some of these metabolites have been reported to accumulate to greater levels than the parent compound in biota (Renoux et al. 2000; Lachance et al. 2004; Belden et al. 2005; Sims and Steevens 2008). At waste sites, TNT long-term stable degradation product–soil complexes may be formed (Achtnich and Lenke 2001; Neuwoehner et al. 2007). Therefore, in assessing the integrated toxicity of all present contaminants, bioassays using relevant bioindicator organisms should be performed in support of chemical analysis (Scott-Fordsmand and Weeks 2000; Robidoux et al. 2002; Lewis et al. 2004; Belden et al. 2005; Neuwoehner et al. 2007; Batzias and Siontorou 2006). In a search for non-mammalian species for evaluating the impact due to anthropogenic compounds released to the terrestrial environment, earthworm is the species of choice (Fugère et al. 1996; Scott-Fordsmand and Weeks 2000). The compost earthworms *Eisenia fetida*/*andrei* have gained recognition as sentinel organisms to monitor soil pollution (OECD 1984; ISO 2008). Biomarkers allow assessment of the effects of sublethal stress to sentinel organisms, and they constitute a group of parameters that could be modified at the molecular, histological, immunological, physiological, organismal, and population or ecosystem levels (Martín-Díaz et al. 2006). The evaluation of cellular biomarkers in earthworms can be used in a predictive way, allowing to set priorities for the initiation of bioremediation strategies.

The comet assay (single-cell gel electrophoresis) is a rapid and sensitive method for the detection of primary DNA damage on the individual cell level, increasingly used in biomedical research, human and environmental bio-monitoring studies, and genotoxicity testing. Since DNA damage may result in severe consequences for individuals, species, and ecosystems, it is regarded as an important indicator to be used in the assessment of earthworm health (Reinecke and Reinecke 2004; Casabé et al. 2007).

Lysosomal membrane integrity appears to be a generic indicator of cellular well-being in eukaryotes. Lysosomes are membrane-limited organelles having a functional role in intracellular digestion and recycling of macro-molecules (e.g., proteins, carbohydrates, lipids) and as a natural inmunological defense system. A lysosomal destabilization biomarker, the neutral red retention time (NRRT), sensitive to a wide range of pollutants, can integrate the combined effects of a complex pollution matrix and has been used in terrestrial ecotoxicity studies (Svendsen and Weeks 1997; Casabé et al. 2007).

Earthworm coelomocytes, the coelomic fluid harboring cells, have properties similar to mammalian leucocytes, are relatively easy to obtain, and may be useful to perform both bioassays on the same biological samples (Weeks and Svendsen 1996; Burch et al. 1999). To narrow down the range for contaminants prior to soil testing and for the comparison of different biomarkers, the filter paper contact test could be a simple and inexpensive research tool (Roberts and Dorough 1984; Robidoux et al. 2002; Gastaldi et al. 2007; Gong et al. 2007).

According to the above token, our aim was to assess in parallel the two biomarkers (NRRT and DNA damage) as indicators of biological effects

in *E. fetida* exposed to sublethal concentrations of TNT.

Materials and methods

Chemicals

TNT (99% pure) was obtained from the Armed Forces Council on Research and Technology (CITEFA, Argentina). Acetonitrile (analytical grade) was from Merck (Argentina). All other reagents were from Sigma (St. Louis, MO, USA).

Exposure tests

Mature adults of *E. fetida* with a well-developed clitellum and a wet mass between 0.40 and 0.60 g were selected from the colony maintained in our laboratory. For bioassays, earthworms were previously washed with dechlorinated tap water and placed on moist filter paper overnight in order to let them empty their guts before the start of the test. Filter paper contact exposure was performed according to OECD (1984) with slight modifications. The filter papers (45 cm^2) were impregnated with a range of TNT solutions in acetonitrile (1 ml) to obtain 0, 0.25, 0.5, 1.0, 2.0, and 4.0 $\mu g/cm^2$ sublethal concentrations. After solvent evaporation, papers were placed individually into vials, lining their inner surface, moistened with 1 ml distilled water, and a single earthworm was introduced into each tube. Tubes were covered with nylon gauze, sealed with a tight fitting rubber and incubated for 24, 48, or 72 h at $22 \pm 2°C$ in continuous darkness. Sets of five worms were exposed to each sublethal concentration, and two independent experiments were performed.

Coelomocytes collection

Coelomic fluid from individual earthworms, containing coelomocytes, was extruded through dorsal pores after stimulation with an electric current. At each time point, organisms were removed, rinsed with dechlorinated tap water, and blotted dry. Each worm was placed in a glass conical tube containing 1.5 ml of phosphate-buffered saline (PBS; Ca^{++}, Mg^{++} free). The worm was subjected to three short bouts of electric stimulation (<1 s) at 6 V DC using two platinum electrodes attached to a 500-mA universal transformer. The diluted coelomic fluid was immediately transferred to an eppendorf tube. Viability was determined in an improved Neubauer hemocytometer diluting 10 µl of cell suspension with 10 µl of 0.4% trypan blue in PBS. Blue stained cells were counted as non-viable. The percentage of viability was calculated based on the percentage of unstained cells.

Comet assay

Comet assay was performed by adaptation of Singh et al. (1988) on coelomic fluid obtained as described above. Immediately after extrusion, 10 µl coelomocyte suspension were mixed with 75 µl 0.75% low melting point (LMP) agarose in PBS at 37°C and spread over a microscope slide precoated with 100 µl 1% normal melting point agarose. After 5 min of solidification at 4°C, a layer of LMP agarose was placed on top and left to harden for 5 min at 4°C. Slides were immersed in alkaline lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% *N*-lauryl sarcosinate, 10% DMSO, and 1% Triton X-100, pH 10) and stored overnight at 4°C. After lysis, slides were rinsed with neutralization buffer (0.4 M Tris–HCl, pH 7.5) and immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA, pH > 13) at 4°C for 12 min, to allow DNA unwinding. Electrophoresis was conducted for 20 min at 25 V (1 V/cm) and a starting current of 250 mA. As internal control for DNA migration, coelomocyte suspension of non-exposed worms was treated in vitro with 50 µM H_2O_2. After electrophoresis, microgels were drained and washed with three changes, 5 min each, of neutralization buffer. Afterwards, they were washed two times with 1 ml distilled water during 5 min. Moisture excess was removed in an oven at 37°C, and slides were stored in a dust-free box until stained with ethidium bromide (20 µg/ml), just prior to microscopic observation. The whole procedure was performed under dim light to minimize artifactual DNA damage.

The slides were independently coded and scored by a single observer. One hundred randomly selected cells (50 cells from each of two

replicate slides) were analyzed from each organism using an Axioplan (Zeiss, West Germany) fluorescent microscope. Cells with damaged DNA display increased migration of DNA fragments from the nucleus, generating a "comet" shape with a head and a tail. Cell nuclei were rated visually and classified into four categories according to the tail intensity (size and shape): class 0 (no damage or <5% DNA in tail), class 1 (low-level damage or 5–30% DNA in tail), class 2 (medium level damage or 30–75% DNA in tail), class 3 (high level damage or more than 75% DNA in tail). Comets with small or non-visible head and large diffuse tails were classified as "clouds" and were not scored (Burlinson et al. 2007). An arbitrary damage index (DI) was used to express the extent of DNA damage and is defined as $\mathrm{DI} = \sum n_i \times i$, where n_i is number of cells with damage class i (0, 1, 2, or 3). Then, the DI for the 100 cell nuclei examined from each worm could range between 0 (undamaged) and 300 (maximally damaged). The percentage of damaged cells (D%) was also calculated.

NRRT assay

In the NRRT test, coelomocytes of the same groups of earthworms utilized for the comet assay were used, but only three earthworms for each group. The NRRT on the coelomocyte cells of each worm was measured according to Weeks and Svendsen (1996) with slight modifications. Coelomic fluid (20 μl) was placed on a microscope slide, and the cells allowed adhering for 60 s prior to the application of 20 μl neutral red working solution (80 μg/ml) and a coverslip. The working neutral red concentration was renewed each hour to avoid crystallization of the nonpolar neutral red in the PBS solution. Each slide was scanned for 2 min at 5-min intervals under a light microscope (×400). In healthy cells, neutral red is retained in the lysosomes, and the cytosol is colorless. For damaged cells, efflux of dye into the cytosol results in redness of the cytosol. The slides were kept in a humidity chamber when not under observation. Only the cells readily adhering to the surface of the microscope slides were observed. Observation was stopped when the number of cells with fully stained cytoplasm reached >50% of the total number of cells counted. This time was recorded as the NRRT.

Statistical analysis

Data were expressed as mean ± SD and analyzed using the Statistica 99 software (Statsoft Inc, Tulsa, USA). To test for differences among treatments and over time, two-way ANOVA and Newman–Keuls' tests were performed. Linear correlations were performed using the Pearson linear correlation test. The probability level of significance was considered $p < 0.05$.

Results

Earthworm mortality and coelomocyte viability

No mortality of the earthworms was recorded during the exposure periods at any tested concentration. The coelomocyte suspension (10 μl) had about 10,000–15,000 cells (determined by cell counts). The viability of coelomocytes was around 90% at all tested TNT concentrations.

Comet assay

A small percentage (<10%) of clouds (extremely damaged cell nuclei) was found on slides corresponding to earthworms exposed 72 h to 2.0 μg/cm^2, and they were not scored. This effect was not observed at lower concentrations. At 4.0 μg/cm^2, the percentage of clouds increased notoriously; therefore, scoring was not performed at this concentration.

Figures 1 and 2 summarize the results of the comet assay, taking into account the above-mentioned considerations. A significant concentration-related increase in the percentage of damaged cells was observed ($r = 0.912$, $r' = 0.871$, $r' = 0.867$ after 24, 48, and 72 h exposure, respectively). This percentage differed significantly when compared with controls ($p < 0.01$), at all assayed TNT concentrations, with exception of 0.25 μg/cm^2 at 72 h (Fig. 1a).

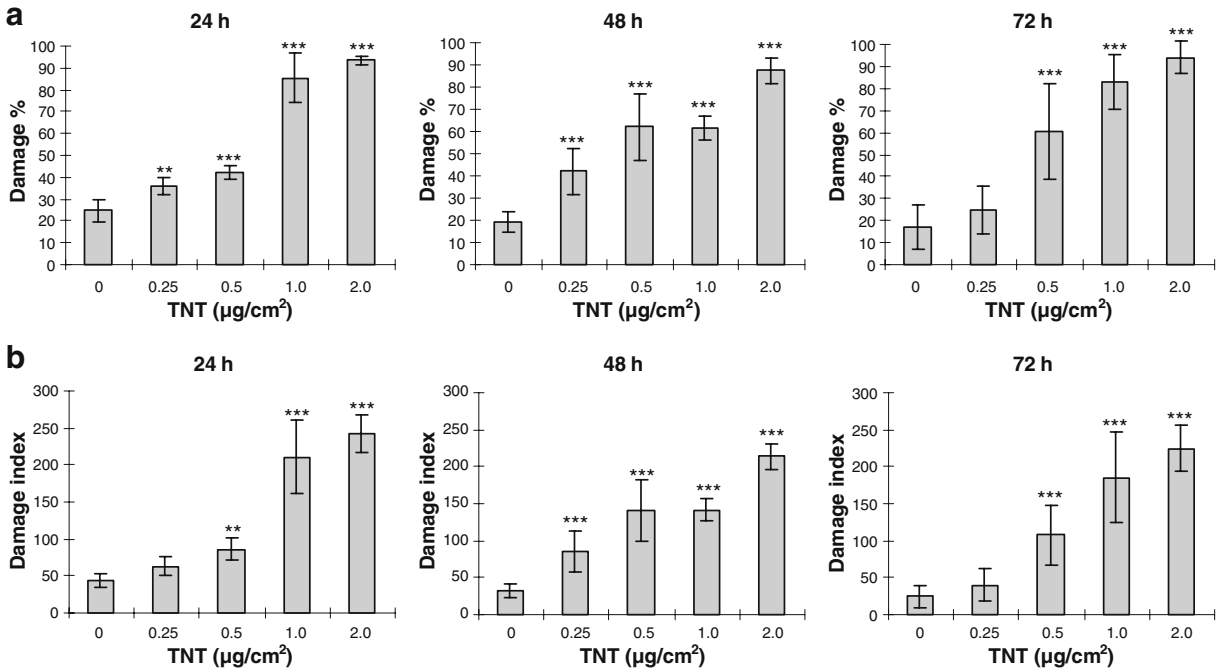

Fig. 1 Damage % **a** and damage index **b** in coelomocytes of *E. fetida* after exposure to TNT at different times. Data are expressed as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$: significant differences with respect to controls

It can be clearly seen that the background level of DNA damage (control) was essentially low at the three time points studied, with 24.8 ± 5.0%, 19.1 ± 4.6%, and 16.9 ± 10.3% of damaged cells.

Figure 1b shows the extent of DNA damage measured as DI, a weighted value of damage, according to scoring of cells. In concordance with the D%, a significant correlation between DI and TNT concentration ($r = 0.897$, $r' = 0.886$, $r' = 0.855$ for 24, 48, and 72 h exposure, respectively) was obtained. TNT concentrations of 0.5, 1.0, and 2.0 μg/cm^2 showed significant differences with controls ($p < 0.01$) at all time points assayed. The DI of the lowest assayed concentration (0.25 μg/cm^2) was not significantly different from control after 24 and 72 h. In vitro treatment of coelomocytes with 50 μM H_2O_2 resulted in a D% of 83.0 ± 8.5 and a DI of 206.0 ± 21.2.

Figure 2 shows the cell distribution in the different damage classes in control and treated groups, after 24, 48, and 72 h exposure. At lower concentrations, the majority of comets fell in the lower damage classes, with an increasing shift to the higher damage classes at the higher concentrations.

When analyzing the damage response along the time of exposure in treated worms, two different patterns (corresponding to low doses and high doses, respectively) were observed. With 0.25 and 0.5 μg/cm^2, there was a high percentage of undamaged cells (class 0), at the three assayed times. When compared to 24 h, at 48 h exposure, the results showed an increase in the percentage of comets at higher damage classes (classes 2 and 3), which was reflected in an increase in D% and DI for these two concentrations (Fig. 1a, b). At 72 h, a shift back to lower damage classes occurred; D% and DI showed a decrease in relation to 48 h values. Instead, a high percentage of highly damaged cell nuclei at 1.0 and 2.0 μg/cm^2 TNT concentrations at the three studied times was observed. This resulted in high values, which remained almost unaltered during the exposure period. DNA migration in coelomocytes of control worms remained constant during the 72-h exposure period. The

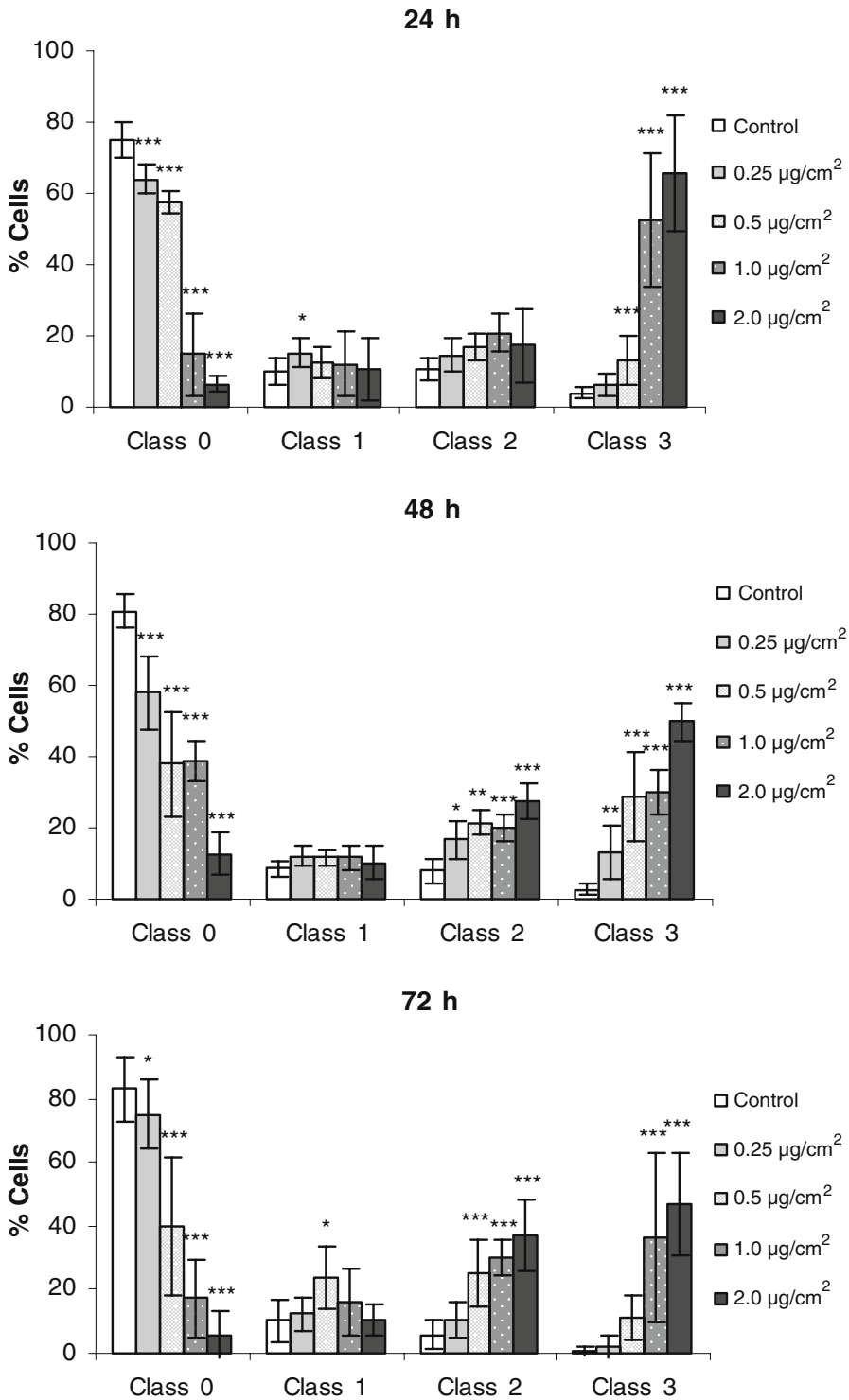

Fig. 2 DNA damage scores. Percentage of cells in each damage class in coelomocytes of *E. fetida*, after TNT exposure at 24, 48, and 72 h. Data are expressed as mean ± SD. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$: significant differences with respect to controls

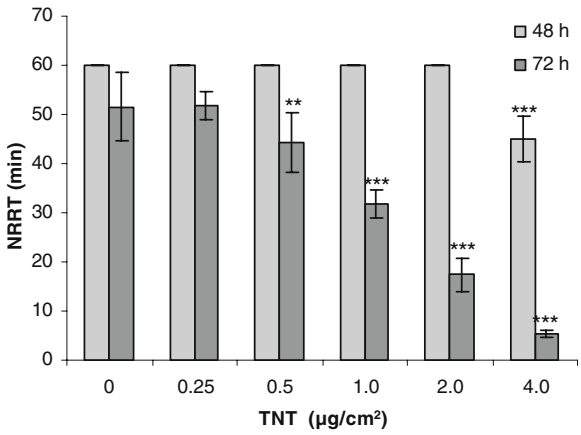

Fig. 3 NRRT in coelomocytes of *E. fetida* after TNT exposure at 48 and 72 h. Data are expressed as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$: significant differences with respect to controls

anomalous data for both D% and DI after 48 h exposure to 1.0 µg/cm^2 could not be explained.

NRRT

Analysis of NRRT results showed a significant interaction between time and treatment (two-way ANOVA, $p < 0.001$). As shown in Fig. 3, at 48 h, the NRRT of coelomocytes of worms exposed to concentrations between 0.25 and 2.0 µg/cm^2 of control worms exceeded 60 min. However, the NRRT significantly diminished (45.5 ± 4.5 min) at 4.0 µg/cm^2. After 72 h exposure, the NRRT of the control group decreased to 51.6 ± 6.9 min. In earthworms exposed to TNT, NRRT presented a concentration-dependent decrease ($r = 0.942$, $p < 0.001$). This decrease was significantly different with respect to control at 0.5, 1.0, 2.0, and 4.0 µg/cm^2 (44.2 ± 6.1, 31.7 ± 2.9, 17.3 ± 3.3 and 5.3 ± 0.8 min, respectively; $p < 0.01$).

Discussion

The purpose of the present work was to evaluate the suitability of two bioassays (comet and NRRT), performed on the same sample, as non-specific and early indicators of biological effects in *E. fetida* exposed to sublethal concentrations of TNT.

We used the filter paper contact test because it offers a prompt and easy method for screening chemicals potentially toxic to earthworms under strictly standard exposure conditions (Roberts and Dorough 1984; Robidoux et al. 2002; Gastaldi et al. 2007; Gong et al. 2007). Renoux et al. (2000) using this contact test found that earthworms of the species *Eisenia andrei* absorb TNT through the body wall, even after a very short contact (about 5 min). They also detected traces of 2-ADNT and 4-ADNT, suggesting that TNT was rapidly transformed to these compounds and subsequently to 2,4-DANT. Although the concentrations of these metabolites represented one third to one half of those observed with earthworms placed in soil, their relative proportion to each other after 24 and 48 h were in accordance with soil observations (Renoux et al. 2000). TNT primary reduction products have been found to be toxic to certain soil invertebrates, such as earthworms (Lachance et al. 2004). Therefore, TNT and its metabolites could have contributed to the observed effects.

No mortality was found during our experiments, indicating that the lowest observable effect concentration was above the tested concentrations in this study.

The coelomocytes play an important role in immune defenses of earthworms (Manerikar et al. 2008). We previously compared different protocols for coelomocyte collection (data not shown). Electrical extrusion was selected because of its rapidity, its better yields in terms of number of cells and viability, and the advantages of getting samples without chemical interference. After the electric shock, earthworms always recovered. Besides, low DNA or lysosomal damage in control cells was obtained with this method.

The single-cell gel electrophoresis or comet assay is a very sensitive method for detecting DNA strand breaks and alkali labile sites in individual cells, by measuring the migration of DNA from immobilized nuclear DNA. The degree of migration is related to DNA damage (Lee and Steinert 2003).

In this work, cells were visually rated into four damage classes. All the scoring of slides was blind, that is without the recorder knowing to which treatment they belonged. Visual scoring methods

are simple, less time-consuming, do not require special software, and allow an appreciation of the relative tail intensity, which itself is a function of break frequency (Guecheva et al. 2001; Kumaravel et al. 2009). According to Burlinson et al. (2007), comets with small or nonexistent head and large and diffuse tails (called "ghost cells" or "clouds") potentially represent necrotic or apoptotic cells. This type of effect confounds the interpretation of the comet assay results and should be excluded. This was the case of our visual observations at 4.0 μg/cm^2; therefore, this concentration was not included in the scoring.

Under the conditions of this experiment, both D% and DI showed significantly DNA damage at almost all concentrations, at all time points assayed, when compared to negative controls. Thus, earthworms contacted and absorbed the test substance. Time was enough for the development of DNA lesions, and the fraction of damaged cells and the intensity of genetic injury were related to the TNT concentration. DNA damage is a transient response; time-dependent variations were considered to result from a balance between damage and repair activities, maintaining DNA breaks within certain limits (Sun et al. 2004; Qiao et al. 2007). Our results obtained at the lower TNT concentrations (0.25–0.5 μg/cm^2) showed an increased DNA migration at 48 h, with a decrease close to initial levels after 72 h exposure. This decrease could be due to the activation of DNA repair system. At the higher assayed concentrations (1.0–2.0 μg/cm^2), the high DNA damage observed remained constant during the 72 h exposure, suggesting that the rate of DNA repair was not enough to compensate such damage. To our knowledge, it is the first time this protocol is applied to evaluate time integrative DNA damage response in *E. fetida* exposed to sublethal TNT concentrations.

According to Lee and Steinert (2003), chemicals associated with DNA damage can be grouped into four classes: (1) chemicals that act directly on DNA; (2) chemicals whose metabolites cause DNA damage; (3) chemicals that cause the production of reactive oxygen species (ROS) that can damage DNA; (4) chemicals that inhibit DNA synthesis and repair. During reductive transformation of TNT to 2-ADNT or 4-ADNT, some reactive intermediates could be generated which may form adducts with the thiol groups of cellular peptides, cellular proteins, and DNA. Although the proportions of metabolites are concentration- and species-dependent, the contribution of covalent binding of intermediate compounds that react with DNA could probably be one mechanism contributing to the TNT-observed genotoxic effects (Renoux et al. 2000; Lachance et al. 2004; Belden et al. 2005; Ownby et al. 2005). Alternatively, it has also been reported that the reduction of TNT is a redox reduction, which produces ROS. Subsequently, the cell has to adapt to the oxidative stress, and this may cause metabolic modifications and DNA damage (Kumagai et al. 2004). DNA damage may also have been produced as consequence of the excision repair enzymes and through the inhibition of DNA synthesis, apoptosis, or necrosis (Lee and Steinert 2003; Ateeq et al. 2005).

Lysosomal compartment is sensitive; one of the characteristic changes is the increased fragility of the lysosomal membrane. Over time, neutral red, a lipophilic, slightly basic dye that is taken up in uninjured cells and accumulated in these organelles, tends to leak out into the cytosol which becomes stained by the dye. NRRT assay is a simple and inexpensive technique, but it requires a trained operator. In this study, all determinations were performed by a single operator, and as in the case of the comet assay, they were blind.

NRRT has already been established as responsive in earthworms exposed to metals, such as copper, cadmium, zinc, lead, organic contaminants like polycyclic aromatic hydrocarbons, energetic compounds, and pesticides like acetochlor, glyphosate, chlorpyrifos, diazinon (Maboeta et al. 2003; Svendsen et al. 2004; Xiao et al. 2006; Casabé et al. 2007; Gastaldi et al. 2007; Maleri et al. 2008). It was also previously established as a useful biomarker of TNT exposure in *E. andrei* (Robidoux et al. 2002). In our study, after 72 h exposure, NRRT of controls fell from >60 min (48 h) to 51.60 ± 6.95 min, suggesting some stress probably due to the longer exposure time. At this time, NRRT showed a decrease with increasing TNT concentration, indicating an increasing damage on lysosome membrane in treated earthworms. Our results are in agreement with those

of Robidoux et al. (2002), which, exposing earthworms *E. andrei* to TNT on filter paper at concentrations that fell into the range used in this work, observed a significant decrease in NRRT at TNT concentrations >1.8 μg/cm^2, with no observable effect concentration of 0.9 μg/cm^2.

Early responses of the lysosomal system are generally thought to provide a first indication of pollutant exposure, since injurious lysosomal reactions frequently precede cell and tissue pathology (Renoux et al. 2000; Moore et al. 2006). With respect to the outcome of the cell after lysosomal rupture, it may be variable. The magnitude of this rupture and consequently the amount of hydrolytic enzymes released into the cytosol may induce reparable sublethal damage, apoptosis, or necrosis (Bursch 2001; Hwang et al. 2004). Hwang et al. (2004) showed that *Crassostrea virginica* oysters recovered their lysosomal health, when they were allowed to eliminate the toxic in a clean environment. The recovery of destabilized lysosomal membrane could be due to: (1) recovery of lysosomal function in damaged cells, as chemicals in the cell decrease; (2) cell regeneration, these healthy regenerated cells replacing damaged cells that are removed as they die.

Biomarkers assayed in this study showed clear responses to sublethal TNT exposure in *E. fetida*. The two methods can be performed on the same sample, providing sensitive unspecific biomarkers of cell injury and DNA damage. Further studies with contaminated soils will be performed in order to validate both bioassays as tools to be included in integrated approaches to assess the toxicity of TNT polluted sites.

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