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# Ecotoxicological evaluation of foundry sands and cosmetic sludges using new earthworm biomarkers

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**Abstract** The management and final disposal of industrial wastes are a matter of considerable human concern. The present study evaluates the cyto/genotoxic effects and changes of the coelomic cell formulas exerted by aqueous leachates and solid waste (SW) of two industrial residues using coelomocytes extruded from *Eisenia fetida*. The assayed wastes corresponded to industrial foundry and cosmetic activities. After 14 days of exposure, we obtained a group of endpoints that reflect the toxicity/genotoxicity, coelomocyte formula and indexes; and the mortality classical value (LC50-14d). Among the variables measured, total coelomocytes formula (eleocytes + amebocytes + granulocytes) appears as a single and easy parameter to assess the toxicity of eluates at short exposure times. We applied a set of assays using earthworms as test organism that would allow evaluating SW as well as its aqueous leachates. It is easy to run trials combining exposures of 1 h to 14 days, which can be integrated into the implementation of the traditional test for evaluating acute toxicity.

**Keywords** Genotoxicity · Earthworms · Cosmetic sludges · Foundry sands · Hazardous wastes

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## Introduction

The final disposal of solid wastes (SWs) is based on whether they are hazardous or non-hazardous. Wastes are defined, as hazardous or not, according to their properties such as: corrosivity, toxicity, ignitability and reactivity. Those that exhibiting one or more of these four characteristics, are considered hazardous. In many countries, SWs are classified, based on the specific levels of a broad range of chemicals. In United States, a waste exhibits the toxicity characteristics if it represents a substantial threat to human health and the environment. Waste toxicity is measured by using the toxicity characteristic leaching procedure (TCLP). However, the ecotoxicological characterization of waste is part of its assessment as hazardous or non-hazardous according to the European waste list. The hazard criteria Ecotoxic (H14) is defined if it may present risks for one or more sectors of the environment. In Argentina, the same above mentioned parameters, are considered to define whether a waste is hazardous or not under both a National 24.051 (SADSN 1992) and Provincial 11.720 laws (OPDS, Buenos Aires 2006). Among them, ecotoxicity is included and it is estimated from the available information on the waste, if not, toxicity tests must be performed using a battery of assays using species belonging to three ecological levels. However, the type of toxicity tests to be used, or whether evaluation should be performed after acute or chronic exposure, for example, are not stated. The criterion H14 'ecotoxic' lacks an assessment and testing strategy. In addition, no specific threshold values have been defined so far (Moser et al. 2011). A literature review conducted over the past 20 years has revealed that the number of publications regarding the ecotoxicological characterization of wastes is increasing but remains limited (Pandard and Römbke 2013).

The analytical techniques used generally for characterization of wastes only measure levels of pollutants. However, physico-chemical parameters or TCLP, do not provide information about the toxic effects of these wastes as a whole. The analysis of toxic effects of the environmental contaminants has employed a wide range of sentinel species, microbes, and individual cells. Earthworms play a major role in physical, chemical and biological process in the soils, and may represent up to 60–80 % of the total soil biomass. They are keystone species within ecosystems having great impacts on decomposition activity, nutrient mineralization and primary production (Edwards and Coulson 1992). They are used extensively as bioindicators of environmental contamination (Calisi et al. 2011). Moreover, earthworms may affect the chemical forms of pollutants (organic and inorganic residues) during food consumption, metabolism, and excretion.

Nowadays, there is an increasing concern in monitoring biomarkers to provide measurements as well as estimations of biological exposure to emergent pollutants. To accomplish this target, several sublethal endpoints for testing both genotoxicity and cytotoxicity have been employed on terrestrial and aquatic organisms, including earthworms. Among them, the induction of DNA single-strand breaks by the single cell gel electrophoresis (SCGE), also called comet assay is one the most frequently used and recommended endpoint for detecting primary DNA damage in nucleated cells (Cotelle and Ferard 1999).

In the case of earthworms, it is possible to evaluate this DNA damage by using SCGE assay a non-invasive biomarker on their coelomic cells or coelomocytes (Di Marzio et al. 2005; Quiao et al. 2007; Han et al. 2014; Wang et al. 2015). Coelomic fluid acts as hydrostatic skeleton and it is in contact between the inner and the outer environment, playing an important role in the homeostasis maintenance, nutritive and innate immunity functions (Cooper 1996). The coelomocytes are characterized by a pronounced polymorphism, been their classification based on the cytomorphometric, ultrastructural and cytochemical properties. There are three major cell types, namely eleocytes (chloragogen cells), hyaline amoebocytes and granular amoebocytes or granulocytes (Adamowicz 2005; Kurek et al. 2007). Furthermore, it has been reported that the proportion of the different cellular types of coemolocytes may be related to health and the immune earthworm response (Wang et al. 2015). Thus changes in cell proportions at the coelomic level in organisms exposed to different xenobiotics and type of stress turn this parameter as a new biomarker of exposure. Several studies support this concept (Homa et al. 2003; Olchawa et al. 2006). In agreement, Adamowicz and Wojtaszek (2001) have shown that eleocytes proportion of *Dendrobaena veneta* decreased after exposure to cadmium and copper.

In this work, we evaluated the acute and chronic eco(geno)toxicity effects exert by two SW produced as sludges in a cosmetic wastewater treatment plant (cosmetic sludge, CS) and sands used in foundry activities (foundry sands, FS). Furthermore, whether their final disposal could create risk for the environment and human health was also analyzed in order to achieve a better understanding of the real environmental risk that exists if these residues are used or disposed indiscriminately.

We evaluated both cytotoxic and genotoxic effects on coelomocytes of the earthworm *Eisenia fetida* (Oligochaeta) exposed both in vivo and ex situ conditions using the SW as a whole or as its aqueous leachates, respectively.

## Materials and methods

### Samples characterization and leachates preparation

SWs were sampled from two industrial areas corresponding to cosmetic and foundry activities. In the case of CS the sample consisted in a composite sample, taken after mechanical dehydration of the secondary sludge. FS were also composite samples taken at random subsamples in the temporary disposal within industrial area. Physico-chemical characterization of these wastes was conducted by measuring pH, water holding capacity (WHC), wet fraction, volatile solids (VS) at 550 °C, total organic carbon (TOC) and physical structure (US EPA 1991; Carter 1993; APHA 1998). Leachates were prepared according to US EPA (1991) recommendations. Briefly, 250 g of waste and 1 L of demineralized water were shaken continuously for 48 h in darkness at room temperature (RT). Physico-chemical parameters of leachates such as pH, ammonium and chemical oxygen demand (COD), were analyzed following method #4500 and #5220 of Standard Methods (APHA 1998). Control soil used corresponds to a natural soil at the experimental field of National University of Lujan, characterized by Di Marzio et al. (2007). Metals were measured in solid samples and leachates according to Method #3111B, whereas As was determined following method #3114C of Standard Methods (APHA 1998). Detection limits were 0.05 mg/L and 0.1 mg/L for As. Spiked soils were recovered at 85–110 %.

A qualitative screening of semi-volatile compounds was performed. These were extracted using the US EPA method 3550C (2007) with some modifications. Briefly, 40 g of each SW, FS and CS, were mixed with 20 g anhydrous sodium sulfate. The samples were extracted with 200 mL of 65:35 acetone–hexane mixture. Each SW and solvents were shaked for 1 h at RT. Then, three cycles of 5 min of ultrasonic treatment at 40 kHz were performed. The extract was separated from the solid sample by

vacuum filtration with filter paper of 0.45 μm. Finally it was concentrated using a rotary evaporator, to a final volume of 1 mL. Two microliters were injected into a Shimadzu gas chromatograph 17A V 1.3 model, with mass spectrometer QP 5050A and MSSolution software. Experimental conditions included 30 m PTE-5 fused-silica capillary column (Supelco, Bellafonte); linear velocity of carrier Helio, 36.2 cm/s, splitless injection mode with sampling time of 4 min and total flow of 11.7 mL/min; a temperature program of 100 °C for 2 min to a final temperature of 280 °C at 10 °C/min and held for 10 min; an injector and capillary interface temperatures of 280 °C. The MS was used in scan mode ranged from 50 to 350 m/z.

## Test organisms

*E. fetida* adults, average wet weight 300 mg, were purchased from local source (Luján, Buenos Aires). Earthworms were maintained in moistened control soil (pH 6.6 ± 0.26, 25 % sand, 48 % slime, 27 % clay, moisture 40–60 % of WHC 60 ± 5 mL/100 g), at RT, under natural photoperiod; fed with 10 % of alfalfa forage. The worms were allowed to acclimate to laboratory conditions for several weeks before testing. Experiments were performed with specimens exposed to concentrations of CS and FS equivalent to 9 and 26 % of LC50-14 day for each SW, respectively.

## Coelomocyte extrusion

A non-invasive extrusion method was used for collecting earthworm coelomocytes according to Di Marzio et al. (2005). The extrusion medium (EM) consisted in 5 % v/v ethanol in saline solution (0.85 % NaCl) and 2.5 mg/mL EDTA, adjusted to pH 7.5. Before extrusion the earthworms were rinsed in tap water at RT and placed on a damp paper towel overnight to allow them to void the contents of their guts. The pooled castings of five organisms was placed into centrifuge tubes containing 2 mL of EM/individual and incubated for 1 min at RT. Coelomic fluid containing the extruded cells was diluted with calcium and magnesium free phosphate buffered saline (PBS), washed twice, and centrifuged at 2000 rpm at 4 °C during 10 min. The final pellets were resuspended in 2 mL of PBS.

## Colelomocyte counts and in vivo and ex situ cytotoxicity

Extruded cells were counted using a counting chamber improved Neubauer hemocytometer. The extruded cells were characterized according to their morphology as eleocytes, amoebocytes or granulocytes according to Adamowicz and Wojtaszek (2001) and Adamowicz (2005). Correlations between the number of each cell type and individual wet weights were determinated in control earthworms reared in the control soil during 140 days. The following endpoints were calculated.

Whole body coelomocytes index (WBCI): log(10) (*Cn/wwf*); where *Cn* is total coelomocytes number average per individual/mL of celomic fluid; and *wwf* is wet weight without feces (WWF). Absolute trophic index earthworm (ATIE): *En/Cn,* where *En* is total eleocytes number average per individual/mL of celomic fluid. Relative trophic index earthworm (RTIE): ATIE/*wwf.*

To evaluate in vitro cytotoxicity, coelomocytes were incubated 1 h at RT, in the following percentage of water leachates: 1.5, 3, 6, 12.5, 25, 50 and 100 %, PBS was used as negative control. The cell viability was expressed as the percentage of viable cells measured with 0.4 % of Trypan blue. One hundred cells were counted on each slide and three replicate slides were analyzed per specimen. For ex situ evaluations, the same protocol was used with extruded cells of earthworms exposed to SW during 14 days. Data were expressed as average of total coelomocytes per individual/mL of celomic fluid.

## Genotoxicity in vivo and ex situ exposures

Solutions of PBS and $H_2O_2$ were used as negative and positive controls, respectively. Ex situ cell exposures were conducted by immersing slides containing the triple-layer agarose gels used for the comet assay in PBS (negative control), hydrogen peroxide (positive control) and waste leachate. The time of exposure was 1 h in all assays in vitro. The in vivo exposure followed the general procedures of the 14 day toxicity test guidelines descried in the "earthworm reproduction test—*E. fetida*" (OECD 2000). Adults with observable clitellum, were exposed to both control soil and soil contaminated with two wastes. One hundred cells were analyzed, for duplicate. The SCGE assay was carried out on cells collected after 7 and 14 days of exposure. For the in vitro exposure, extruded coelomocytes were exposed in slides to 100 μM of $H_2O_2$ as positive control, to PBS as negative control. The leachate's concentrations for genotoxicity were chosen based on cytotoxicity results; corresponding to the LC50-1h and LC50-1h/2. DNA migration was measured as % tail DNA in all assays.

## Single cell electrophoresis (SCGE) assay

SCGE assay protocol proposed in Di Marzio et al. (2005) was used. Assays were performed under indirect incandescent light at 4 °C. Gels were composed of three layers of agarose. The suspensions of earthworm's cells were diluted (1:2) with 1 % low-melting-point agarose (LMPA)

giving a final agarose solution of 0.66 % and 80 µL of the cell suspension were transferred to a slide having a thin layer of solidified 0.5 % agarose. The slides were covered with a coverslip and left on ice for 10 min to allow the second layer of agarose to solidify. The coverslip was gently removed, and 80 µL of 0.5 % LMPA were spread over the second layer. A coverslip was placed on top of third layer and the agarose solidified. This last coverslip was removed and each slide was immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM $Na_2$-EDTA. 10 mM Tris (pH 10) 1 % N-laurylsarcosinate, 1 % Triton X-100 and 10 % dimethylsulfoxide (DMSO) to remove proteins and lipids, during 10 min. Slides were then placed in an electrophoresis tank and covered with electrophoresis buffer (300 mM NaOH, 1 mM $Na_2$EDTA, pH 13.5) for 25 min at RT to allow unwinding. Electrophoresis (300 mA, 30 min, 1 V/cm) was then performed in the some buffer. The slides were washed once, for 10 min in the neutralization buffer (0.4 M Tris, pH 7.5). Before analysis, the slides were stained with 30 µL of 20 µg/mL ethidium bromide. The image of nucleoid were analyzed with microscope Nikon, Eclipse 600, provided with epifluorescence (541–560 nm excitation filter and 590 nm emission filter) linked to an image analysis system (Image Pro Plus, V4.0, Media Cybernetics, MD, USA). The images obtained in the experiments were analyzed with the software program CASP (Końca et al. 2003). Tail DNA %, was used as final genotoxicity endpoint. % Tail DNA comet assay parameter was chosen as it is not measured in arbitrary units, being more meaningful and advisable for regulatory purposes and for inter-laboratory comparisons (Kumarravel and Jha 2006).

### Statistical analyses

EC50 values and 95 % confidence limits from cytotoxicity assay were estimated using Probit analysis software V1.5 (US EPA 2002). Cytotoxicity responses were evaluated by one-way statistical analysis of variance (ANOVA) in conjunction with Tukey's test. LOEC for leachates were obtained by ANOVA in conjunction with Dunnet's test, using ToxStat V3.5 (West Inc. 1996). Cyto and genotoxicity data were analyzed, using R (2013) and Statistica V8 (2007), by non-parametric Kruskal–Wallis, median and Dunn tests (Zar 2010; Sparks 2000).

## Results

### Chemical analysis

Chemical parameters for the assayed SWs are indicated in Table 1. Both wastes differ in their content of TOC and WHC values. For the case of FS, iron and lead were the more abundant quantified metals; in contrast with zinc and nickel for the CS. Chemical analyses of aqueous leachates are shown in Table 2. Cosmetic leachate was characterized by high DQO and ionized ammonia values, as well as high aluminium concentration. By the other hand, FS leachate was mainly characterized, for its acidic condition and high level of lead. The qualitative screening of organic compounds in CS have evidenced the presence of the following compounds, between parenthesis the chemical abstracts service (CAS) number: diacetone alcohol (123-42-2), cyclohexanol dodecyl (55000-30-1), propanal (123-38-6), pentadecanolide (106-02-5), tetradecanol (112-72-1), decamethyl cyclopentasiloxane (541-02-6), nonadecane (629-92-5), pentadecanone trimethyl (502-69-2). Qualitative analysis for FS showed the compound *para-tert*-butylbenzoic acid PTBB (98-73-7) that is an ingredient used in the formulations of binder agents based on alkyd resins.

**Table 1** Physico-chemical parameters and metal concentrations of SWs expressed in mg/kg dry weight

| | Foundry | Cosmetic | | Foundry | Cosmetic |
|---|---|---|---|---|---|
| pH | 6.8 | 6.0 | Cr | 6.11 | 6.19 |
| VS | 1.3 | 9.1 | Pb | 205.2 | 3.38 |
| TOC | 4.84 | 61.81 | As | >0.1 | >0.1 |
| WHC | 25.02 | 73.34 | Cu | 4.47 | 12.85 |
| Total Fe | 2813.74 | 86.48 | Zn | 38.9 | 394.5 |
| Al | >0.3 | >9 | Ni | 4.25 | 22.11 |

VS in %

*TOC* total organic carbon, expressed in mg C/g, *WHC* water holding capacity in mL of water/100 g

**Table 2** Physico-chemical parameters and metal concentrations of aqueous leachates

| | Foundry | Cosmetic | | Foundry | Cosmetic |
|---|---|---|---|---|---|
| pH | 4.32 | 7.71 | Total Cr | 0.05 | 0.5 |
| $NH_4$ | 21.2 | 212 | Pb | 5 | 0.15 |
| TOC | 13.7 | 722.4 | As | 0.10 | 0.10 |
| COD | 299 | 1157.5 | Cu | 0.09 | 0.25 |
| Total Fe | 1.2 | 0.24 | Zn | 0.10 | 3.0 |
| Al | 0.3 | 9.0 | Ni | 0.04 | 0.33 |

All values expressed in mg/L

*TOC* total organic carbon, *COD* chemical oxygen demand

### Extrusion cell, viability and count cell for ex situ assays

The viability of extruded coelomocytes used in the ex situ experiences, measured with Trypan blue, exceeded 90 % in

all cases. The evaluation of total coelomocytes populations indicated that eleocytes were the most abundant cell type, followed by amoebocytes and granulocytes. The relative average proportion of eleocytes:amoebocytes:granulocytes, or "coelomocytes formula" was 60:30:10. The eleocytes were majority group, coinciding with the proportion obtained by Di Marzio et al. (2005) in *E. fetida* and Eyambe et al. (1991) in *L. terrestris.*

## In vivo and ex situ cytotoxicity and coelomocyte formulas

In Fig. 1 is indicated the time-dependent variation of coelomocyte proportions of acclimated earthworms at lab culture under control conditions in relation to their wet WWF.

The EC50-1h and EC50-14d values for coelomocyte toxicity are indicated in Table 3. These values were obtained considering the exposure for 1 h of extruded cells or ex situ conditions to aqueous leachates, and the viabilities of the cells which were extruded from exposed earthworms directly to the SWs during 14 d. The EC50-1h value for CS leachate was higher than that for FS leachate, indicating lower toxicity. This aspect is opposite when we compare the effects after 14 days of exposure to the waste as a whole.

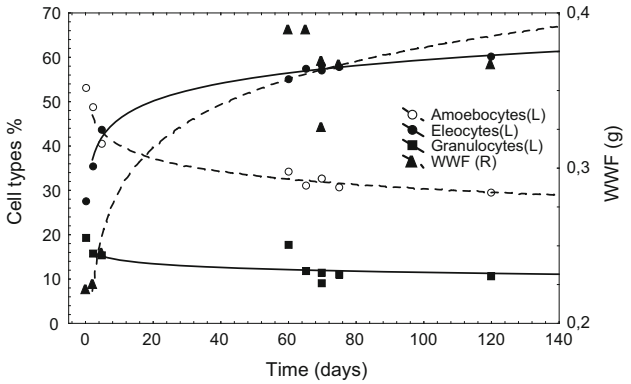


**Fig. 1** Coelomocyte percentages and wet weight without faeces (WWF) variations in relation to culture time at lab control conditions

**Table 3** Cytotoxicity endpoints and confidence limits (95 %) on total coelomocytes obtained from ex situ and in vivo exposures at 1 h and 14 days, respectively

| | EC50 (%) | LOEC (%) |
|---|---|---|
| EC50-1h ex situ | | |
| Foundry sand leachate | 2.12 (0.62–7.22) | 1.5 |
| Cosmetic sludge leachate | 9.15 (6.98–11.88) | 1.5 |
| EC50-14d in vivo | | |
| Foundry sand | 31.41 (19.49–83.55) | 13 |
| Cosmetic sludge | 6.91 (4.84–12.03) | 4.5 |

By the other hand, data obtained from in vivo and ex situ conditions showed similar toxicity for CS. However for FS the EC50-1h and 14d, were different at almost one order of magnitude, which may be indicating an adaptative response of the earthworms to the residue FS.

The data analysis showed that all concentration of the assayed leachates were significant statistically with respect to the control ($p < 0.01$). In the case of FS leachates, four groups differ from each other (ANOVA—Tukey, $p < 0.05$). One group is formed for 1.5, 3 and 12 %, the second group is formed for 6 and 50 %, and separately 25 and 100 %. For the cosmetic leachate also were formed four groups, 1.5 has been separated of the 12 and of 25 %. A linear concentration–response (Pearson coefficient, 0.978) was obtained for the cosmetic leachate, contrary to foundry leachates. If we analyze the toxicity responses taking into account cell types, results showed that amoebocytes and eleocytes showed higher mortalities at higher concentrations ($p < 0.01$). Differently, granulocytes have same response independently of the percentage of leachates. The coelomocyte formulas and indexes changed during in vivo exposure as showed in Table 4. The WBCI, that considers the total coelomocytes number did not change comparing treatments and control, at 7 days of exposure; and decrease at 14 days for earthworms exposed to 26 % of FS and 4.5 and 9 % of CS. Granulocytes densities have been constant in all samples. On the contrary, ATIE and RTIE that take into account the total number of eleocytes, decreased for both residues and exposure times, reducing the amount of nutritive cells to the internal tissues. Amoebocytes were increased likely as a physiological response to the chemical stress, and it was more evident for earthworms exposed during 14 days to CS.

## SCGE assay following ex situ exposure

FS and cosmetic leachates were genotoxics after 1 h of exposure of the coelomocytes to these water elutions. Concerning DNA damage, measured as % of tail DNA were statistically different with respect to PBS solutions or negative controls at dilution as low as 1.06 and 4.57 % for FS and CS respectively (Fig. 2).

## SCGE assay following in vivo exposure

DNA damage after 7 days of exposure, all the evaluated concentrations were statistically different with respect to the control soil for both wastes (Fig. 3). In the case of exposure to SWs during 14 days, the surviving earthworms showed high coelomocytes mortalities, allowing to measure DNA damage only at the lower concentrations, 2.6 % for FS and 0.9 % for CS. In both cases the DNA migration

**Table 4** The population structures of total coelomocytes at 7 and 14 days after in vivo exposure

| | Day 7 | | | Day 14 | | |
|---|---|---|---|---|---|---|
| | ATIE | RTIE | WBCI | ATIE | RTIE | WBCI |
| Foundry sand (%) | | | | | | |
| 0 | 0.59 (0.03) | 1.49 (0.12) | 6.13 (0.02) | 0.59 (0.03) | 1.49 (0.12) | 6.13 (0.02) |
| 2.6 | 0.65 (0.01)* | 1.56 (0.37) | 6.11 (0.09) | 0.56 (0.03) | 1.21 (0.12)* | 6.12 (0.02) |
| 13 | 0.56 (0.015)* | 1.35 (0.12) | 6.08 (0.03) | 0.56 (0.013) | 1.38 (0.11)* | 6.08 (0.03) |
| 26 | 0.52 (0.023)* | 1.31 (0.18)* | 6.03 (0.08) | 0.52 (0.019)* | 1.22 (0.044)* | 6.01 (0.05)* |
| Cosmetic sludge (%) | | | | | | |
| 0 | 0.62 (0.01) | 1.46 (0.024) | 6.11 (0.02) | 0.62 (0.013) | 1.45 (0.024) | 6.11 (0.02) |
| 0.9 | 0.66 (0.03)* | 1.48 (0.056) | 6.06 (0.03) | 0.48 (0.018)* | 1.11 (0.14)* | 6.08 (0.02) |
| 4.5 | 0.65 (0.03)* | 1.25 (0.064)* | 6.09 (0.12) | 0.51 (0.013)* | 1.14 (0.031)* | 6.05 (0.03)* |
| 9 | 0.53 (0.017)* | 1.41 (0.087) | 6.09 (0.02) | 0.49 (0.006)* | 1.25 (0.22)* | 6.01 (0.02)* |

Quantified as *ATIE* absolute trophic index earthworm, *RTIE* relative trophic index earthworm, *WBCI* whole body coelomocytes index

* $p < 0.001$ (Kruskal–Wallis one way analysis of variance on ranks)

was statistically different with respect to control the control worms (Fig. 4).

## Discussion

The assayed wastes were toxics on *E. fetida* considering the cell viability, DNA damage, and relative coelomocyte indexes. These coelomocytes, extruded from healthy worms, were exposed directly to aqueous elutions for 1 h, or they were obtained from healthy worms that first were exposed for 7 and 14 days to the SWs. The absolute and relative trophic indexes were sensitive, as final endpoints, to evaluate short and longer exposure times. These take in account the density of eleocyte cells, which are mainly related with nutritive and immune functions. They are chloragocyte-derived that are floating freely in the coelomic fluid, and contain significant riboflavin levels that has an adaptive value for worms vulnerable to soil-derived

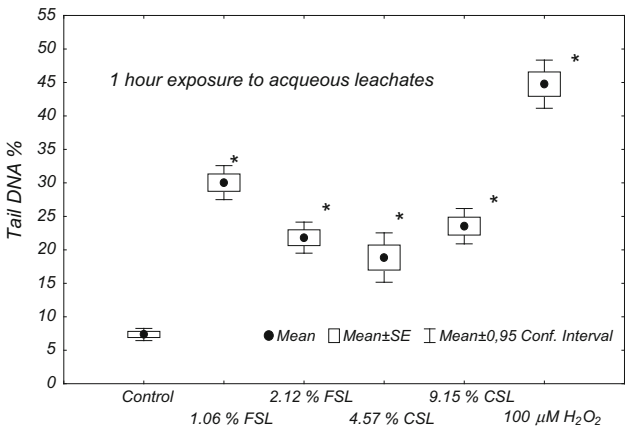


**Fig. 2** Genotoxicity of foundry sand (FS) and cosmetic sludge (CS) leachates, and hydrogen peroxide. DNA damage measured as tail DNA (%) in coelomocytes exposed ex situ during 1 h. *$p < 0.001$ Kruskal–Wallis *U* one way analysis of variance on ranks

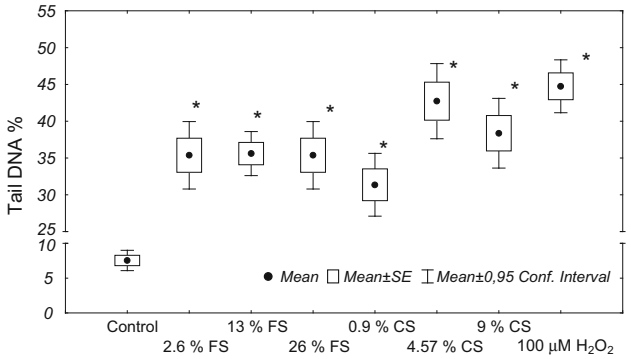


**Fig. 3** Genotoxicity of foundry sand (FS) and cosmetic sludge (CS) and hydrogen peroxide. DNA damage measured as tail DNA (%) in coelomocytes exposed in vivo during 7 days. *$p < 0.001$ Kruskal–Wallis *U* one way analysis of variance on ranks

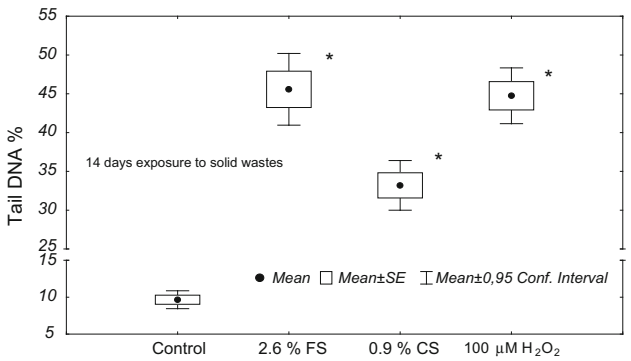


**Fig. 4** Genotoxicity of foundry sand (FS) and cosmetic sludge (CS) and hydrogen peroxide. DNA damage measured as tail DNA (%) in coelomocytes exposed in vivo during 14 days. *$p < 0.001$ Kruskal–Wallis *U* one way analysis of variance on ranks

pathogen invasion. This suggests that riboflavin plays an important role in immunity of lumbricid worms, as it does in vertebrates (Mazur et al. 2011).

Both CS and FS had metals in their solid matrix; the main difference was for the case of FS its high concentrations of iron and lead. The residue CS presented a variety of organic compounds that might exert their toxic effect by polar and non-polar narcosis. Substances found in cosmetic wastewater belong to the group of the pharmaceutical and personal care products which includes drug and cosmetic ingredients, that in many cases are toxic to humans and the environment (Esplugas et al. 2007; Reif et al. 2008; Volpe et al. 2012). Among these compounds both organic and inorganic could be present. Organics include hydrocarbons, proteins, ethers, esters, aldehydes, ketones, alcohols, carboxylic acids, and also more complex derivatives thereof and products of various biochemical transformations (Bogacki et al. 2011). Inorganics include acids, hydroxides, salts, oxides and heavy metal compounds (Kasprzyk-Hordern et al. 2009). Substances such as grease and mineral oils, organic dyes and surfactants are also common in all cosmetics wastewaters. In the case of FS the compound *tert*-butylbenzoic acid, an ingredient used in the formulations of binder agents based on alkyd resins, was determined by GC–MS (Carnin et al. 2012). By the other hand, the waste foundry sands (WFS) are generated in the molding of metal parts. Approximately 10 million tons of spent foundry sands (FS) are produced in the United States each year, and their beneficial use in agricultural and horticultural applications is being considered (Dungan et al. 2009). In Argentina, there are approximately 300 foundries, producing approximately 200,000 tons of waste sands per year. The molding sands are used to make metalcasting and cores, and are combinations of virgin sand and binding agents such as bentonite or organic resin among others (Dungan et al. 2009; Miguel et al. 2012). In the casting process, molding sands are recycled and reused multiple times. Eventually the recycled sands degrade to the point that they can not longer be reused in the casting process and are removed as waste. Although most of the WFS from this process are land filled, there is great interest in diverting them for use in agricultural and geotechnical applications (Carnin et al. 2012). Miguel et al. (2012) concluded that because of the low metal concentrations found, in most WFS examined, it is likely they could be beneficially used in both encapsulated and unencapsulated applications without detriment to human and environment health. These wastes would have potential to be used in the landfill, agricultural and geotechnical applications and would not generate a risk to human health or the environment (Miguel et al. 2013). At least for FS there are results or scientific data that would support this purpose mainly based on toxicity characteristics leaching procedure. However, in these studies ecotoxicity and even less, effect on biomarkers or the potential to exert genotoxicity, was not evaluated. There have been very limited experiences with testing wastes in ecotoxicology (Kostka-Rick 2004; Römbke et al. 2009).

The presence of metals and organics in CS and mainly metals for FS, could explain the differences observed in the toxicological responses comparing both residues. Assessing the values of the different endpoints, for the case of FS, its toxicity was inverse to the exposure time, which may reflect the development of resistance to the toxicity exerted by the metals present in its matrix. Brulle et al. (2006) demonstrate the induction of synthesis of metal-like fixing metallothionein proteins after 14 h of exposure of healthy earthworms, to cadmium spiked soils; and that induction was maximal after 6 days of treatment. The toxic response in the case of CS was at random considering the exposure time (1 h to 14 days), but for most endpoints the toxicity values were very similar regardless of the period of exposure (Fig. 5). Although this residue, besides organic substances had metals such as chromium, zinc or nickel, the type of response observed may be related to the mode of action of narcotic substances. The effects of these compounds are usually evident in the first few hours or days of exposure (Rand 1995). This situation could affect the use of a single set of endpoints that can be used to estimate toxicities at longer exposure times, at least using earthworms as test organisms.

Our results highlighted the need to integrate as many response variables as possible in order to incorporate their implications for risk analysis studies related to final wastes disposal. Acute toxicity and reproduction tests using earthworms are two of the recommended methods that were included in the international ring test organized by the German Environment Agency based on the guidelines of CEN 14735 (2005). The use of biotests for the determination of waste properties potentially hazardous to the environment can also be applied in waste risk assessment. In addition, their ecotoxicological characterization gives information which can be used for their environmental risk assessment (Moser and Kessler 2009). Precisely in order to define suitable test methods for the biological assessment of waste and waste eluates (Römbke et al. 2009). It was performed with three representative waste types: ash from an incineration plant mainly contaminated with heavy metals, soil container containing high concentrations of organic contaminants and preserved wood waste contaminated with copper and other heavy metals. It was recommended that a worm (i.e. oligochaete) test should be part of the final test set as these are in many soils the most important invertebrates, which are also often quite sensitive due to their close interaction with the pore water as well as

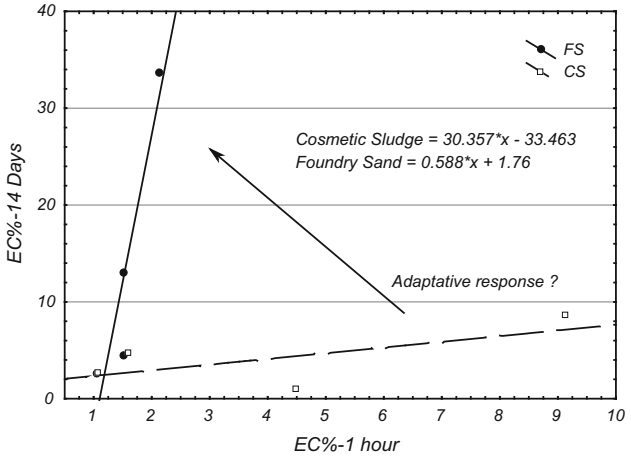


**Fig. 5** Relationship between effective concentrations (EC) at 14 days and 1 h exposure times to aqueous leachates, considering as response variables: cytotoxicity, genotoxicity, and coelomocytes formula of *E. fetida*

the substrate itself (feeding). Because of the low sensitivity of the acute endpoint mortality, alternative methods with more sensitive endpoints have to be checked as the chronic earthworm reproduction test (ISO 1998). However, the application of the latter tests is complicated because it has duration of 56 days against 14 days of acute exposure. Besides the greater sensitivity of this assay is relative because in the mentioned study, chronic rates were between three and five times lower compared to the acute EC50 values. The results of our work include testing periods between 1 h and 14 days, and the relationship between the toxicity endpoints obtained for both waste was higher than one order of magnitude, and also these would allow elucidate mechanisms of adaptation or resistance, to be considered when defining the threshold or acceptable values for their final disposal. Recent studies (Pandard et al. 2006; Wilke et al. 2008; Moser and Römbke 2009) showed that ecotoxicological characterization of waste can be applied satisfactorily on all types of waste (liquid, solid, sludge). The results obtained demonstrated that the response of ecotoxicity tests can vary significantly depending on the type of waste and therefore confirm that this biological approach is suitable to discriminate between ecotoxic and non-ecotoxic wastes. In this study we present a group of tests to evaluate the toxicity of both SW and its aqueous eluates, using in both cases a representative species of terrestrial ecosystems. It is possible evaluate the toxicity of the elutions in 1 h compared to 48 or 96 h, if the *Daphnia* sp and algae tests are performed, respectively. Moreover with this test battery and always using a representative soil species as test organism, also is possible to evaluate genotoxicity of both eluates and solid residues. Furthermore after 14 days of exposure, we can get through a group of endpoints that reflect the toxicity/genotoxicity, coelomocyte formula and indexes; and the mortality classical value (LC50-14d). Among the variables measured, total coelomocytes formula (eleocytes + amebocytes + granulocytes) appears as a single and easy parameter, to assess the toxicity of eluates at short exposure times. This approach gets more and more acceptance in general, but so far mainly for eluate testing (Deprez et al. 2012). Di Marzio et al. (2005) have demonstrated that the evaluation of DNA damage in eleocytes from *E. fetida* using the SCGE assay, is a useful biomarker for the genotoxic effects of chronic exposure to landfilled soils. Since these measurements are made with an intact animal system, the responses reflect toxics bioavailability, bioaccumulation, and biotransformation processes. In full accordance with Pandard and Römbke (2013) the results of biotests are reliable and allow to discriminate between ecotoxic and non-ecotoxic wastes, also are applicable to a very wide range of waste materials (e.g., solid, liquid, multiphasic wastes, sludges).

## Conclusions

Both SWs were toxics to individuals of *E. fetida*. These toxic effects were determined using traditional lethality tests, coelomocyte population counts and biomarkers. In the last case, the evaluation of 1 h genotoxicity, on direct aqueous leachates using coelomocytes, seems be a suitable assay to get rapid information to be used together with or as an alternative to generate toxicological data instead of the more expensive and time-consuming 14 days mortality tests. Also the cytotoxicity on coelomocyte populations and ATIE, RTIE endpoints are very simple methods that resulted sensitive. However, the predictive power of the combinations of these endpoints could change for SWs with different chemicals composition. Final disposal of FS and CS could exert real risk to human and environmental health, thus they cannot be used or disposed indiscriminately.

We applied a set of assays, using earthworms as test organisms, that would allow to evaluate SW as well as its aqueous leachates. It is easy to run trials combining exposures of 1 h to 14 days, which can be integrated into the implementation of the traditional test for evaluating acute toxicity.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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