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Genotoxicity of Acqueous Elutions of Industrial Soils

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Abstract The genotoxicity of industrial soils was evaluated. A single cell electrophoresis assay or comet assay, using eleocyte cells of *Eisenia foetida*, was performed to assess the genotoxicity of aqueous elutions. These were obtained from industrial soils containing metals. All soil samples meet the environmental quality guidelines for metal concentrations. However, elutions have produced genotoxic effects at dilutions as low as 6%. Total metal concentrations for each aqueous elution could express synergistic effects of these compounds.

Keywords Genotoxicity · Metals · Comet assay

During the year 2004, industrial hazardous waste production in Argentina was near to 71.109 t (SADS 2005). The final disposition of these solid wastes is defined according to the chemical compositions that might potentially be present in the samples, in relation to the industrial activity that produced them. The same criteria are used for soils removed from industrial areas, determining their final application according to metals concentration thresholds. Thus, soils could be used for agricultural, residential or industrial landfill, according to the guidelines indicated in Table 1.

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In this work, we studied the genotoxic effects of water elutions from industrial soils. Our purpose was to demonstrate that samples may meet the soil quality guidelines, yet could be environmentally hazardous because of their genotoxic potential.

Materials and Methods

We have worked with 53 soil samples removed from an industrial area subjected to chemical control, to define their final disposition as agricultural, residential or industrial land filling.

Samples were taken from removed industrial soils. All soils meet the concentration limits to dispose of them as residential or agricultural landfill. The sampling area was characterized for same soil type classified according to USDA (2003); it was described for pH, water holding capacity, wet fraction, total organic carbon (TOC) and physical structure, following Carter (1993). Cadmium, lead, nickel and chromium were measured in each soil sample elution following method # 3111 and mercury was analyzed according to method # 3112B of Standard Methods (APHA 1998). Detection limits for chromium and nickel were 0.02, lead 0.05, zinc 0.005, cadmium 0.002 and mercury 0.00034, expressed as μg/L. Spiked soils were recovered at 95%.

Eluates were prepared from industrial soils to evaluate the mobility of chemical constituents in hazardous wastes. Site samples were mixed with 4 mL of calcium- and magnesium-free phosphate-buffered saline (PBS) per gram (dry weight) soil. The slurry was then mixing in total darkness for 48 h at 20 ± 2°C. After mixing, the resulting eluate was centrifuged and then filtered through a 0.45 μm glass fibre filter. Original sample moisture is incorporated into the eluate sample during its preparation. Hence,

Table 1 Soils quality guidelines of metals concentrations (Ley 24051 1993)

Chemical	C A S	Agricultural	Residential	Industrial
Cadmium	7,440–43–9	3	5	20
Chromium	7,440–47–3	750	250	800
Mercury	7,439–97–6	0.8	2	20
Nickel	7,440–02–0	150	100	500
Lead	7,439–92–1	375	500	1,000

Values expressed in μg/g dry weight

a constant "solute/solvent" ratio is assured during the extraction of any site sample.

Genotoxicity of these elutions was evaluated on Eisenia foetida as follows. Adults, with an average wet weight of 290 mg, were purchased from a local source (Luján, Argentina). They were maintained in moistened control soil at room temperature (RT), and under a natural photoperiod, fed with dry high-protein baby cereal. After purchase, the worms were allowed to acclimatize to laboratory conditions for several weeks before testing. Coelomocyte extrusion in guaiacol glycerol ether-free medium, a modification of the non-invasive extrusion technique of Eyambe et al. (1991), was used for collecting earthworm coelomocytes. The modified EM consisted of 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 individuals were placed into centrifuge tubes containing 3 mL of EM/individual and incubated for 2 min at RT. The earthworms were returned to their culture medium (soil). Coelomic fluid containing the extruded cells was diluted (1:3) with calcium- and magnesium-free phosphate-buffered saline (PBS), washed twice and centrifuged at 150*g* and 4°C. The final pellets were resuspended in 2 mL of PBS. Coelomocyte counts and viability evaluation of extruded cells from 20 animals were counted using a counting chamber similar to an improved Neubauer haemocytometer. The extruded cells were characterized according to their morphology as eleocytes, amoebocytes or granulocytes (Adamowicz and Wojtaszek 2001). Viability was expressed as the percentage of viable cells measured with 0.4% of trypan blue. Single cell electrophoresis assay (SCEA) was carried out according to Di Marzio et al. (2005). Basically, the gels were composed of three layers of agarose. The suspensions of earthworm 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 1% 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 this 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_2EDTA, 10 mM Tris (pH 10), 1% *N*-laurylsarcosinate, 1% Triton X-100 and 10% dimethylsulfoxide (DMSO)) to remove proteins and lipids. Lysis time was 10 min. Slides were then placed in an electrophoresis tank and covered with electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.5) for 25 min at RT to allow DNA unwinding. Electrophoresis (300 mA, 30 min, 1 V/cm) was then performed in the same buffer. The slides were washed once, for 10 min, in neutralization buffer (0.4 M Tris, pH 7.5). Before analysis, the slides were stained with 30 μL of 20-mg/mL ethidium bromide. The slides were observed using an epifluorescence microscope (Nikon Eclipse 600) with a dichroic filter (excitation filter, BP 510–550 nm; suppression filter, BA 590 nm) linked to an image analysis system (Image-Pro Plus V4.0, Media Cybernetics, Maryland, USA). DNA migration was measured as: % Tail DNA (the percentage of DNA that migrated from the nucleus in the direction of the anode expressed as 100-head % DNA). Hydrogen peroxide (H_2O_2) and PBS were used as positive and negative controls, respectively.

In vitro cell exposures were conducted by immersing slides containing the triple-layer agarose gels used for the SCGE assay in several soil elution concentrations, H2O2 and PBS for 60 min. Before lysis, some slides were stained with Trypan blue to evaluate cell viability. Lysis, unwinding and electrophoresis were then performed as described above. Experiments were performed in triplicate, and 900 eleocytes were analyzed per each soil. Eleocytes were identified by their larger size (comet head) relative to the other cell types (Di Marzio et al. 2005).

A square root transformation was applied to Tail DNA (%) data to stabilize the variances and approximate a normal distribution. Lowest LOEC values for evaluated elutions were obtained by one-way statistical analysis of variance in conjunction with Dunnet's test, using ToxStat V 3.5 (West Inc. 1996). Soil samples were analyzed by principal component analysis (PCA) to explore the degree of correlation of genotoxicity responses (LOEC) with the final concentrations of metals. One matrix was constructed using this variable and the 53 soil samples as the horizontal rows of the PCA data matrix. The PCA was performed using StatGraphics Plus software V 2.1.

Results and Discussion

The assayed soils were Mollisols of the suborder Argiudolls subgroup typic Argiudolls. Clay–silt–sand content was 17.02, 57.41, 25.50%, respectively. Mollisols are commonly the very dark coloured, humus-rich surface

Table 2 WHC, water holding capacity (mL/100 g); TOC, total organic carbon g/kg; MF, moisture fraction

	pH	TOC	WHC	MF
Mean	6.32	4.02	57.36	0.13
Standard deviation	0.36	3.96	5.49	0.05
Coefficient of variation (%)	5.64	98.88	9.57	39.12

horizon in which bivalent cations are dominant on the exchange complex and the grade of structure is weak to string. These are the mineral soils of the steppes (USDA 1999, 2003).

The average composition is within the standard characteristics of those soils of the Pampas: pH, 6.1 ± 0.26; organic carbon (g/kg), 19.3 ± 4.80; available P (mg/kg), 26.8 ± 8.24; total nitrogen (g/kg), 1.7 ± 0.42; clay content (%), 28.5 ± ?4.93; silt content (%), 48.5 ± 8.25; sand content (%), 23.0 ± 4.09; cation exchange capacity (cm^3/kg), 30.3 ± 5.37 (Lavado et al. 2005). Table 2 shows critical parameters of the assayed samples, which conditioned metal elutions.

Metal concentrations of elutions made with these soils are indicated in Table 3.

Two components of PCA analysis (Fig. 1) have explained more than 70% of variance. The first component would define the variability, taking into account the total metal concentrations for all samples or as a size variable which can be interpreted as a measure of overall metal load. The second component separated two or three soil groups according to the pairs Pb–Cd, Cr–Hg and Ni. The genotoxicity of the samples showed a great variability and seems to be related mainly with the concentrations of mercury and chromium.

Results with positive control H_2O_2 of extruded coelomcytes fell within expected values: significant increases in DNA migration (% Tail DNA) occurred at concentrations between 37 and 300 μM ($p < 0.01$) with the extent of migration plateaued H_2O_2 at a concentration above 37 μM. At least for LOEC concentrations of total metals to each elution, citotoxicity effects measured using Trypan blue dye were not different from cell controls (ANOVA–Dunnet; $p < 0.05$).

Table 3 Metal concentrations for each soil elutions

Sample	Pb	Cr	Hg	Cd	Ni	Ge	Sample	Pb	Cr	Hg	Cd	Ni	Ge
# 1	44	16	0.11	0.7	13	12	# 28	12	14	0.06	0.66	9.6	25
# 2	27	15	0.05	0.51	15	25	# 29	28	18	0.19	0.71	10	12
# 3	40	17	0.33	0.61	13	12	# 30	39	33	0.69	0.71	19	6
# 4	13	15	0.17	0.7	10	25	# 31	44	13	0.16	0.48	11	25
# 5	8.9	12	0.03	0.7	14	25	# 32	72	21	1.8	0.76	14	1
# 6	29	9	0.01	0.48	14	25	# 33	15	17	0.07	0.29	36	25
# 7	19	15	0.14	0.48	14	12	# 34	120	25	0.41	0.67	15	25
# 8	13	14	0.05	0.41	15	25	# 35	13	16	0.13	0.53	9.3	20
# 9	179	25	0.42	1	25	12	# 36	14	17	0.09	0.56	10	25
# 10	18	18	0.13	0.71	14	6	# 37	12	17	0.11	0.53	12	12
# 11	104	17	0.07	0.81	11	12	# 38	12	18	0.13	0.69	11	25
# 12	356	7.3	0.03	0.72	13	12	# 39	14	13	0.7	0.35	7.8	35
# 13	44	13	0.08	0.72	13	12	# 40	12	13	0.08	0.52	10	35
# 14	17	14	0.12	0.51	15	25	# 41	17	13	0.04	0.71	11	25
# 15	11	14	0.07	0.41	13	25	# 42	12	14	0.04	0.61	13	25
# 16	41	15	0.16	0.7	13	12	# 43	72	15	0.69	0.71	17	6
# 17	11	30	0.03	0.81	12	12	# 44	25	17	0.08	0.29	37	20
# 18	16	9	0.03	0.48	11	12	# 45	19	19	0.1	0.41	33	20
# 19	20	15	0.07	0.72	15	12	# 46	55	15	0.23	0.47	11	25
# 20	32	14	0.11	0.82	12	12	# 47	24	16	0.07	0.42	11	15
# 21	27	15	0.63	0.71	18	12	# 48	12	20	0.07	0.56	11	20
# 22	18	18	0.19	0.35	37	25	# 49	14	19	0.15	0.44	12	25
# 23	20	20	0.11	0.88	16	6	# 50	11	16	0.04	0.54	8.7	25
# 24	20	15	0.29	0.18	11	25	# 51	14	14	0.03	0.71	11	25
# 25	30	18	0.11	0.36	11	25	# 52	39	33	0.69	0.71	19	6
# 26	33	16	0.05	0.64	19	12	# 53	21	14	0.08	0.61	12	25
# 27	12	16	0.36	0.42	8.9	25							

Cr is expressed as total chromium, *Ge* LOEC genotoxicity measured as percentage (%) of water elutions. All in μg/L

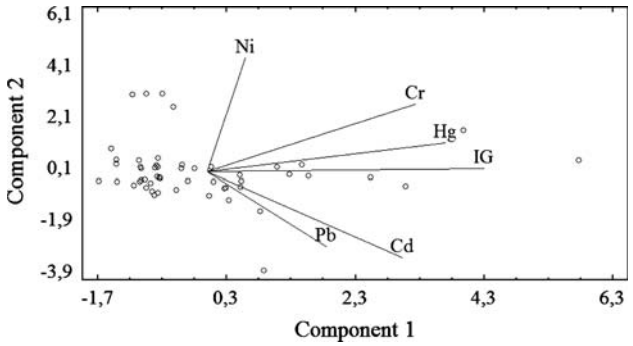

Fig. 1 Biplot of two-dimensional scattergram corresponding to the distributions of PC parameters of each soil and its genotoxicity. The two components explained more than 70% of total variance

The assayed metals seem to have lower genotoxic effects when these are measured separately, mercury and chromium being the more powerful genotoxics with respect to cadmium, lead and nickel. This could be shown in the PCA analysis. Silva-Pereira et al. (2005) exposed human leukocytes in vitro at low concentrations of mercury. They found that genotoxicity started at 0.1 mg/L. Cadmium may directly damage DNA through the induction of single-strand breaks (Hassoun and Stohs 1996), or damage DNA indirectly by inhibiting DNA-repair enzymes (Hartwig 1998). Bierkens et al. (1998) exposed earthworms to natural soils spiked with up to 1,000 mg Cd/kg. Using the SCGE assay, they observed increased genotoxicity in the coelomocytes. They found recoverable Cd concentrations of 976 mg/kg and 6 mg/L for the soil and elution samples, respectively. Di Marzio et al. (2005) found that eleocytes from *E. foetida*, exposed for 60 min to 50 µM $CdCl_2$, had significant increases in DNA migration ($p < 0.01$). Reinecke and Reinecke (2004) exposed in vitro coelomocytes from *E. foetida* to 2, 6, and 12 mg/L of nickel. They found that these concentrations caused the formation of comets of which the mean tail lengths differed significantly from those of unexposed controls but not from each other. The results indicated DNA single-strand breaks in soil invertebrate cells caused by exposure to a nickel compound, verifying previous findings for mammals which indicated that this metal has genotoxic potential. These results therefore suggest that earthworms may be useful indicator organisms to assess the genotoxic risks of nickel pollution to terrestrial environments and that the comet assay is a useful tool to use as a biomarker of genotoxic effects on invertebrates in soil. Also, Wozniak and Blasiak (2004) indicated that nickel, at a very low, non-cytotoxic concentration of 59 µg/L might interfere with two different steps of DNA repair after DNA impairment for other genotoxic agents.

Lee et al. (2004) indicated that physiologically relevant, nanomolar chromium concentrations (5.2 µg/L) cause DNA base oxidation in human white blood cells in vitro as assessed by the modified comet assay. Wozniak and Blasiak (2003) used the alkaline comet assay to evaluate genotoxicity of lead on isolated human lymphocytes. They found that lead concentrations, at between 0.2 and 20 mg/L, induced DNA damage. However, data were confused, since at 0.2 and 2 mg/L they observed an increase in the tail length, whereas at 20 mg/L a decrease was seen.

We performed a relation of total metal concentrations (TMC) for each aqueous elution which could express synergistic effects of these metals, as shown in Fig. 2. It indicates that an increase of one order of magnitude for TMC showed an equivalent increase in the genotoxic power of assayed elutions.

The final fate of industrial soil samples must take into consideration the chemical characterization of the whole sample and the genotoxicity potential of water elutions by using the SCGEA protocol with coelomocytes of *E. foeida*, as a reliable and economic methodology.

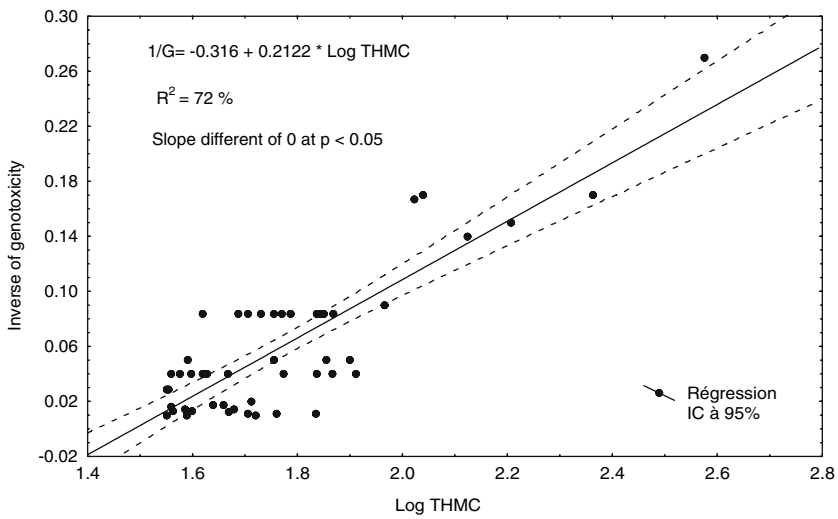

Fig. 2 Relation between total metal concentrations (TMC) for each soil sample and the inverse of genotoxicity LOEC

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