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Ecotoxicological Characterization of a Disposal Lagoon from a Munition Plant*

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Residues of munition factories have become a serious worldwide environmental concern due to their hazardous effects on a variety of biota, and the latent threat of leaching through the soil and polluting groundwater (Green et al. 1999; Hawari et al. 1999; Simini et al. 1995). It is currently accepted that the ecotoxicological impact of soil/sediment contaminants under field conditions cannot be assessed only by chemical methods, since toxicity is closely related to the bioavailability of the components. Bioavailability depends on the spatial variability of soil composition and the temporal variation due to the partition and diffusion of contaminants deeper into soil particles. In addition, it is well known that at munition waste sites, highly toxic and complex chemical mixtures may be composed not only by the original compounds, but also by their breakdown products (Kalafut et al. 1998; Kendall et al. 1996; Robidoux et al. 1999).

An ideal protocol to characterize soil toxicity is not available yet (Schouten et al 2000). Bioassays can be useful to elucidate the integrated ecotoxicological effects of environmental contaminants (Siciliano et al. 2000). The earthworm acute toxicity test measures the lethality of soils to *Eisenia* sp. in a 14-day static bioassay (ISO 1993). This test has an advantage over other bioassays because it exposes the organisms to the unaltered solid matrix of the contaminant, and thus it involves only the use of the directly bioavailable toxicant in the test material (Jarvis et al. 1998). Earthworm survival has been previously used to establish the ecological risk of munition manufacturing plants (Simini et al. 1995). Indigenous soil microorganisms are also important bioindicators of soil pollution due to their role in the maintenance of soil fertility, intimate contact with the soil microenvironment, and rapid response to environmental contamination (Gong et al. 1999). An enormous effort is being devoted to optimize remediation protocols for contaminated soils, sediments, sludges and waste lagoons. *In situ* bioremediation is an attractive and cost-effective clean-up strategy. It has been used successfully for the treatment of hydrocarbon polluted sites (Saterbak et al. 2000). As regards nitrocompounds, there is a considerable interest on their final location and on the release of potential metabolites in the environment. Recent studies indicated that the aerobic degradation of nitrocompounds might exhibit the metabolic potential necessary to address its environmental remediation (Kalafut et al. 1998).

The aim of this work was to characterize the profile of pollution of sediments at

*The authors dedicate this manuscript to the memory of the late Dr. Edgardo J. Wood who was the major impetus behind this multidisciplinary project

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an unlined wastewater disposal lagoon of a munition plant located in Argentina, which has been operating for fifty years, and to initiate studies on the feasibility of bioremediation. As a result of the operations carried out in the plant, not only explosive residues but also acid from the munition production, and petroleum compounds would reach the lagoon. This multidisciplinary study includes: counts of aerobic heterotroph microorganisms, assessment of the toxicity to earthworms of the species *Eisenia fetida*, and chemical determination of total petroleum hydrocarbons (TPH), pH, 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (DNT) and nitrocellulose (NC). Some preliminary studies on the optimization of soil conditions for bioremediation, such as water content, pH correction, and addition of nutrients are also reported.

MATERIALS AND METHODS

TNT and DNT were obtained from the Armed Forces Council on Research and Technology (CITEFA). Both were more than 99% pure. All other reagents and solvents were of analytical grade.

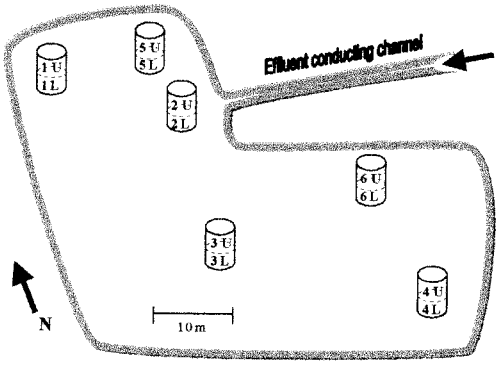

Figure 1: Position of core sampling locations within the wastewater lagoon. Numbers and letters separated by a dashed circle corresponde to core samples taken in the upper (U) and lower (L) layers below the sediment-water interface.

Samples were collected on March 11, 1999 (end of the summer season in this sub-tropical country) from an unlined wastewater lagoon with an area of about 5000 m^2 situated within the boundaries of the munition factory. It was surrounded by an earthen hedge of about 1.8 m. Its depth varied from 0.80 m at its mouth to about 1.65 m. Sediment cores (50 cm deep) –including sludge and subsoil– were obtained at six different locations of the lagoon (Figure 1) with an Augers device. Each core was sectioned into 0 to 25, and 25 to 50 cm layers –named upper (U) and lower (L) samples, respectively– representing the depths below the sediment-water interface.

Thus, the sediment samples available for this study were 1U, 1L, 2U, 2L, 3U, 3L, 4U, 4L, 5U, 5L, 6U and 6L. Two soil samples from a nonpolluted similar geographical area were collected to be used as controls. All samples were placed in plastic bags, stored in coolers, and transported to the laboratory immediately after sampling. Sampling tools were washed and dried between each sample collection. Samples were dried in air at room temperature to a constant weight, homogenized thoroughly, and passed through a 2 mm sieve. After sieving and prior to microbial assays, samples were stored in the dark at 25°C; samples for chemical assays were kept refrigerated. Each sample was extracted and analyzed in triplicate. All assays were completed within 3 weeks of sampling.

To determine TNT and DNT, 2.0 g samples were extracted in dichloromethane (Noguera and Freedman 1996), using an ultrasonic bath. The solvent was evaporated under nitrogen and the extract was dissolved in methanol. Aliquots of the extracts were directly subjected to HPLC (Shimadzu LC-6A) with UV detection at 240 nm. A 150 x 4.6 mm column filled with 5 µm ODS was used; the isocratic mobile phase was a mixture of methanol/water (60:40) at a flow of 1 mL/min. The limit of detection of the method was 0.5 mg/kg for TNT and 1.0 mg/kg for DNT.

Total petroleum hydrocarbon (TPH) index was quantitated using a Nicolet 520P FT-IR with Omnic software. Samples of 3.0 g were extracted in carbon tetrachloride, according to APHA-AWWA-WPCF (1995) with slight modifications. Polar compounds were removed using a silica gel column. The following parameters were used: wavenumber range 2700-3200 cm^{-1}, peak location 2930 cm^{-1}, spectral resolution 4 cm^{-1}, absorption path length 1 cm. The standard reference was prepared mixing n-hexadecane, isooctane and chlorobenzene (15:15:10), and diluting it to the working range (2-40 mg). The limit of detection of the method was 0.2 g/kg. The pH was determined in 1:5 (sediment/soil: double-deionized water) slurries after shaking vigorously for 5 min and waiting for at least 2 hr (ISO 1994). For the determination of nitrocellulose (NC), samples were treated with acetone. Aliquots were filtered, evaporated and weighed. The limit of detection of the method was 0.5 g/kg. Results correspond to nitrocellulose with aromatic nitrocompounds and some hydrocarbons soluble in acetone.

Mature adults of *Eisenia fetida* with a well-developed clitellum and a wet mass between 300 and 600 mg were selected from the colony maintained in our laboratory since 1997. Assays were performed according to the ISO guidelines (1993) with slight modifications. The earthworms selected for the assay were previously washed with dechlorinated tap water and put on moist filter paper for 3 hr in order to let them empty their guts. Afterwards, six earthworms were placed in glass jars containing sediments or control soil equivalent to 500 g (dry weight). Beakers were covered with nylon gauze sealed with a tight fitting rubber band, and soil moisture was maintained with adequate humidity to ensure the survival of the earthworms (60-100% total capacity of retention of water). Mortality was registered daily during 14 days.

For total heterotrophic microoorganism determinations, 1.0 g of each sample was suspended in 9 mL of sterile saline water. Samples were mechanically shaken during 10 min and, after a 1 min stand, the upper liquid phase was collected. Then, 10-fold serial dilutions in saline were made, and 100-µl samples of each one were platted on tryptone soy broth agar (TSBA) according to Lilley and Bayley (1997), and incubated at 32°C for 48 h. Results were expressed as colony forming units per gram of dry sediment (CFU/g *d.s.*). The limit of detection was $1.0x10^3$ CFU/g *ds*. Control samples were processed to compare their data with those of the lagoon samples.

For bioremediation assays, 10.0 g sediments (1L and 4L) were moistened with 10 mL of different solutions: water (*W*); 15 mM potassium phosphate buffer pH 7 (*W+P*); and the same buffer plus the commercial fertilizer Nitrofoska (produced

Table 1. Mean *(SD)* of sediment sample parameters.

Sediment sample*	pH	% Mortality[1] earthworms	Microbial biomass[2] CFU/g *d.s.*	TNT mg/kg	DNT mg/kg	NC g/kg	TPH g/kg
1U	6.60 *(0.33)*	0 *(0)*	$1.2x10^4$ *($4x10^3$)*	280 *(84)*	ND	602 *(120)*	100 *(25)*
1L	6.30 *(0.30)*	33.4 *(16.7)*	$1.5x10^3$ *($1x10^3$)*	430 *(138)*	200 *(60)*	658 *(145)*	313 *(35)*
2U	6.15 *(0.31)*	0 *(0)*	$1.0x10^3$ *($1x10^3$)*	770 *(269)*	150 *(42)*	812 *(203)*	22 *(5)*
2L	6.34 *(0.32)*	0 *(0)*	$1.0x10^3$ *($1x10^3$)*	220 *(62)*	140 *(43)*	889 *(213)*	11 *(3)*
3U	5.30 *(0.27)*	16.7 *(16.7)*	ND	ND	81 *(27)*	856 *(163)*	26 *(8)*
3L	4.70 *(0.24)*	0 *(0)*	ND	ND	ND	820 *(172)*	12 *(4)*
4U	4.64 *(0.22)*	0 *(0)*	ND	38 *(11)*	48 *(12)*	13 *(3)*	1 *(0.2)*
4L	3.88 *(0.20)*	0 *(0)*	ND	7 *(2)*	10 *(3)*	8 *(2)*	ND
5U	6.15 *(0.30)*	100 *(0)*	$5.4x10^4$ *($3x10^3$)*	800 *(270)*	340 *(74)*	355 *(75)*	229 *(66)*
5L	6.13 *(0.30)*	100 *(0)*	$1.32x10^4$ *($2x10^3$)*	250 *(72)*	ND	700 *(140)*	111 *(29)*
6U	4.86 *(0.25)*	100 *(0)*	ND	ND	ND	410 *(86)*	61 *(17)*
6L	4.84 *(0.23)*	100 *(0)*	$9.5x10^3$ *($2x10^3$)*	260 *(80)*	ND	663 *(130)*	102 *(26)*

* Each sample was subdivided into two layers upper (U) and lower (L).

ND: not detected (see Materials and Methods)

CFU/g *d.s.*: colony forming unit / g dry sediment

[1] Control mortality= 0%

[2] Microbial biomass of control soil = $5x10^6$ CFU/g

by BASF Co, and containing 10 g N, 2 g P, 6 g K, and oligoelements such as Mg, B, Fe, Mn, Cu, Zn, Mo and Co per 100 mL) in a proportion 99:1, buffer/fertilizer ($W+P+N$). Afterwards, sediment samples were incubated at 32 °C in an humid chamber. Total heterotrophic microorganisms were determined at 0, 7 and 14 days of incubation. TPH levels were assessed at 0 and 14 days.

RESULTS AND DISCUSSION

TPH levels were between 0 and 313 g/kg, with five samples exhibiting values above 100 g/kg (Table 1). TNT was present in concentrations between 0 and 800 mg/kg. Although these levels were several orders lower than those of TPH, TNT should be considered an important pollutant due to its intrinsic toxicity and to the possibility of forming breakdown products even more toxic than its parent substance (Noguera and Freedman 1996). The pH of sediment samples varied between 3.88 and 6.60, and reflected water currents and a SW-oriented slope in the bottom of the lagoon. Acids were not retained by the sediments and, therefore, had greater mobility and reached deeper areas. Indeed, at location 4, the water level was 1.65 m and sample 4L had a pH 3.88, the lowest registered in the study. It is worth mentioning that all the organic pollutants analyzed showed negligible levels in both samples of this location (4U and 4L). In contrast, TPH levels were higher than 100 g/kg in samples 1U, 1L, 5U, 5L and 6L and higher than 50 g/kg in 6U. TNT also presented the highest concentrations (more than 220 mg/kg) in the same samples, except in sample 6U where it was not detected. Nitrocellulose showed even higher concentrations than TPH (8 to 889 g/kg), although it is considered a compound of low toxicity (OSHA 1992). These results would suggest that hydrocarbons and other organic molecules were retained in sediments by the matrix, and therefore considerable amounts of them remained near their entrance in the lagoon.

Concerning the biological assays used, the impact on the earthworm survival in the 14-d acute toxicity test is clearly observed. All of the earthworms survived in the control soils and even in acidic samples with low chemical contamination (e.g. samples 4U and 4L, see Table 1). In samples with higher chemical levels, earthworms died (100% mortality in samples 5U, 5L, 6U and 6L). Few studies provided specific statistical correlations between bioassay endpoints and soil contamination. In the case of hydrocarbons, most of these studies showed that hydrocarbon composition and soil were important to predict toxicological responses. According to Saterbak et al (1999), survival of earthworms of the species *Eisenia andrei* in a 14-d assay in soils with a TPH content higher than 10 g/kg (quantitated using a GC method) is expected to decrease. As regards TNT, forest soil spiked with this explosive was lethal *to E. fetida* at 150 mg/kg, but a much higher concentration was needed to cause lethality in an artificial soil, where no toxicity was detected at 240 mg/kg (Robidoux et al. 1999). DNT, which is also a toxic compound (Noguera and Freedman 1996), was detected in seven of the twelve samples analyzed.

The sensitivity of the earthworm assay was low when compared with the microbiological assay. Bioassays using soil microorganisms are thought to have relevance to field responses (Gong et al. 1999). Effects on counts of microbial communities from subsurface soil have been demonstrated in studies using

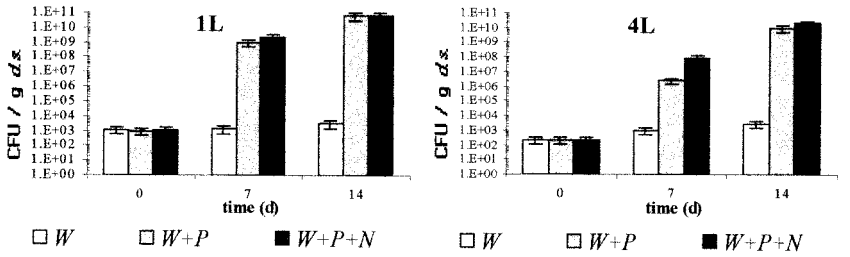

Figure 2: Results of microscale bioremediation experiments in sediment samples. *W*: water; *W+P*: water + buffer; *W+P+N*: water + buffer + fertilizer.

nitrocompounds and petroleum hydrocarbons (Gong et al. 1999; Tarradellas 1997). In unpolluted control soil with pH above 5.5, counts up to 10^6 CFU/g were generally observed, but in polluted samples two to three orders less counts were obtained. Although no control sediment can ideally match test sediment (Lacey et al. 1999), these studies are useful to estimate the overall toxicity of the sample, and thus they would serve as a sensitive biological parameter of toxicity.

However, at pH below 5.5, the acidic stress *per se* was severe enough (e.g. samples 3U, 3L, 4U, 4L, 6U and 6L, Table 1) to cause a decrease in the number of CFU/g, which was very low and did not give any evidence concerning the presence of toxic chemicals. In this case, the earthworm assay appeared as a better indicator.

Figure 2 shows the results of the microscale bioremediation assays performed. As represented in sample 1L, which contained the highest TPH concentration, and sample 4L, which showed the lowest pH, biomass increased along with the time, except in water samples (columns *W*). Columns *W+P* show the effect of the addition of phosphate buffer, supplying phosphorus and buffer capacity; columns *W+P+N*, the combined effect of the inclusion of the buffer and a commercial fertilizer containing nitrogen and oligoelements. These results suggest that the growth of endogenous flora in the samples was essentially limited by the low phosphorus content. The maximum biomass in these nutrient conditions was nearly 10^{10}-10^{11} CFU/g. It is noteworthy that biomass increased faster in sample 1L with an initial pH of 6.3 than in sample 4L with an initial pH of 3.88 (columns *W+P* and *W+P+N* after 7-d incubation). In sample 1L, the biomass increase correlated with a 50% decrease in TPH concentration (from 313 to 154 g/kg in columns *W+P+N*). These results contrasted with the negligible reduction of TPH levels seen when the sample was incubated with water alone. It is well known that microbial degradation of fuel oil or petroleum hydrocarbons is a natural ongoing process. In this screening study, only TPH were quantitated, but we do not know the amounts and types of petroleum hydrocarbons present (Saterbak et al. 1999).

In summary, the lagoon exhibited a spatial heterogeneity in the concentrations of the pollutants analyzed. These, together with the breakdown products formed and the soil matrix properties would generate a different toxicity pattern at each of the locations studied. The pollution plume reached the area underneath the first 25

cm, but further studies should be carried out to determine the depth of the actually contaminated soil. It is worth mentioning that the inclusion of sublethal endpoints such as the effects on the reproduction, induction or depression of some enzyme activities could improve the sensitivity of earthworm bioassays (Oneto et al. 1999; Robidoux et al. 1999; Walker et al. 1992). The presence of microorganisms, even in low numbers, in all sediment samples, including those with low pH and high toxicity, suggested that an indigenous consortium of microflora might be isolated and incremented to be used in the remediation of the polluted area. Nevertheless, more thorough research on the effects of bioremediation on living organisms, as well as on the chemical transformations involved (e. g. the possibility of forming more toxic metabolites) and the kinetics of the process will be necessary before applying this corrective technique to the field.

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