Environmental Monitoring and Assessment (2006) **114:** 169–189
DOI: 10.1007/s10661-006-2523-1

BIOACCUMULATION OF TRACE ELEMENTS IN A WILD GRASS THREE YEARS AFTER THE AZNALCÓLLAR MINE SPILL (SOUTH SPAIN)

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(Received 26 October 2004; accepted 17 February 2005)

Abstract. In this paper, we surveyed the concentration of nine trace elements (As, Cd, Cu, Fe, Mn, Ni, Pb, Tl and Zn) in bermudagrass (*Cynodon dactylon*) 3 years after the mine spill in Aznalcóllar (south Spain). The results were compared with those that had been obtained for the same species in a previous study, 18 months after the accident. Three types of soil condition were determined: i) unaffected soils (UN, control); ii) cleaned up and amended soils (A, amended); and (iii) non-amended soils, inaccessible to the clean-up and remediation operations (NA, non-amended). The trace element concentrations in the plants were lower than those reported in the first sampling for both washed (plant tissues) and unwashed plants (as consumed by herbivores). Apart from Cd, trace elements concentrations (plant tissues) were similar in the A and the UN soils. In the NA soils, the Cd, Fe, Mn, Zn and Pb levels in unwashed plants were excessive for animal consumption. This wild grass seems to be suitable as a soil stabilizer for spill affected soils and as a biomonitor for soil pollution by some trace elements (As, Cu and Zn); however, its potential for phytoextraction is negligible.

Keywords: *Cynodon dactylon*, Guadiamar river, heavy metals

1. Introduction

Mine degraded soils are man-made habitats which experience a wide range of problems when establishing and maintaining vegetation. The metal species commonly found in the soil as a result of human activity include Cu, Pb, Zn, Ni, Co, Hg and Cd. Although some of these metals are required in small amounts by living organisms for their normal physiological functioning, excessive accumulation is toxic to most life forms (Saxena *et al.*, 1999). The problems of heavy metal toxicity are further aggravated by the persistence of the metals in the environment. For example, Pb can persist in the environment for 150–5000 years. There is therefore a pressing need to deal with the problem of excess metal already present in the soil and to prevent future contamination (Saxena *et al.*, 1999).

The restoration of a dense vegetation cover is possibly the most useful and widespread method to physically stabilize mine wastes and reduce metal pollution effects (Bargagli, 1998). Restoration of a vegetation cover can fulfill the aims of stabilization, pollution control, visual improvement and the removal of threats to human beings (Wong, 2003).

In April 1998, a mine accident at Aznalcóllar (SW Spain) affected a total of 4286 ha along the Agrio and Guadiamar river valleys, which was flooded with a volume of ca. $6 \times 10^6\,m^3$ of slurry (for a review, see the special issue in Grimalt *et al.*, 1999). To mitigate the mine accident a large-scale restoration project was launched, including the compulsory purchase of the land (formerly devoted to crops and pastures) and the design of a public nature reserve: 'Green Corridor' (CMA, 2001).

An emergency soil clean up operation quickly started after the mine spill. The toxic sludge covering the ground and a major portion of the contaminated soil surface were mechanically removed and disposed off in an open pit mine. In the more accessible areas (e.g., former croplands), a partial soil restoration was carried out by adding organic matter (up to $20\,t\,ha^{-1}$, CMA, 2001) and calcium-rich amendments. However, despite these clean-ups and partial restoration of the soils, the affected zone was still polluted consistently by trace metals with a fairly irregular distribution (Moreno *et al.*, 2001). Soil pollution was particularly high in zones where cleaning machinery could not operate, especially near the river channel.

The partial restoration of the soil fertility helped to establish a herbaceous vegetation cover, which included bermudagrass (*Cynodon dactylon*), a species which is favored by soil disturbance, such as that derived from the clean-up operations in the Guadiamar basin. *Cynodon dactylon* is an autochthonous grass species, which is well adapted to the local conditions and relatively tolerant to high metal concentration in soils. *Cynodon* is a widespread creeping grass, which may tolerate pollution by trace elements (up to $30{,}000\,mg\,kg^{-1}$ of As in soil), and is useful for stabilizing spill-affected soils (Smith *et al.*, 1998).

Phytostabilization and phytoextraction are the techniques possibly most used in phytoremediation. Phytostabilization can result from either physical or chemical effects. Roots can induce chemical changes to specific metals, which result in their becoming less bioavailable. Also, the most widespread physical phytostabilization arises from the role of the root system, which helps to bind the soil (Pulford and Watson, 2003). On the other hand phytoextraction reduces soil metal concentration by a high capacity for accumulating metal in harvestable root and shoots. Plants used for this purpose should ideally combine high metal accumulation and high biomass production (Salt *et al.*, 1995; Saxena *et al.*, 1999; Barceló and Poschenrieder, 2003).

During the process of vegetation restoration, a continuous monitoring of trace element uptake and allocation in plants must be carried out in order to regulate and avoid, as much as possible, a consistent transfer of these trace elements along terrestrial food chains.

At present, the spill-affected area belongs to a public nature reserve, acting as a 'Green Corridor' between the lowlands (Doñana National Park) and the mountains (Sierra Morena) (CMA, 2003). The successful management of the 'Green Corridor' depends on the immobilization of trace elements still present in the affected soils, so that the afforestations (trees and shrubs) and the herbaceous plant cover growing on them are not a continuous source of toxic trace elements for the trophic web.

In a previous study, carried out 18 months after the Aznalcóllar accident (Madejón *et al*., 2002), it was reported that the tissues of *Cynodon* reached a toxic level of Cd in the amended soils; while toxic levels of Pb, and to a lesser extent of As and Fe, were reached in the unwashed plants. Here, we studied the accumulation of trace elements and nutrients in *Cynodon* growing on soils with different degrees of clean-up and remediation, 44 months after the accident, in the same affected area.

This work forms part of a larger research project in which a number of species (shrubs, trees and herbaceous plants) are periodically monitored in the affected area. Present paper deals with the potential use of *Cynodon* for biomonitorization and phytoremediation. Data on the temporal evolution of trace elements in representative species, *Cynodon* included, are a useful index of the trend followed by the more toxic elements in the affected area, and their possible implications for the trophic web.

2. Material and Methods

2.1. Grass species

A wild grass species abundant in the affected area was selected for the study: *Cynodon dactylon* (L.) Pers. var. *affinis* (Caro and Sánchez) Romero Zarco (bermudagrass). Nomenclature follows Valdés *et al*. (1987). This plant is a warm-season prostate perennial grass that spreads by scaly rhizomes and flat stolons to form a dense resilient turf, with stems up to 30 cm, leaves up to 6 cm and spikes of 1 to 5 cm (Tutin *et al*., 1980; Newman, 1992).

Although considered the world's weediest grass, this plant thrives mainly under soil disturbance, like in ploughed croplands, and rarely invades natural grasslands or forest vegetation. This species requires high temperatures and a lot of sunlight, and is extremely drought tolerant, although moisture significantly increases its growth rate. It tolerates a wide range of soil types and conditions; growth is greater on heavy clay soils than on sandy soils, and on alkaline soils rather than acidic ones. A large amount of available N is required for maximal above-ground growth (Newman, 1992).

2.2. Study area

The upper part of the Guadiamar Basin, in southern Spain, is located in the pyritic mining belt, which has been exploited for copper and other ores since Roman times (ca. 2000 years ago). The large-scale pollution episode (Aznalcóllar accident) had a major ecological impact because the Guadiamar River discharges into the Guadalquivir marshes of Doñana national Park, which is a wildlife site of international importance and a wintering area for many European water birds (Madejón *et al*., 2004).

Soils in the Guadiamar floodplain are mostly neutral or slightly alkaline, with the exception of some terraces (on the right bank), which are rich in gravel deposits and have a low pH. Soil texture is varied, from loamy sand to silty clay. See Cabrera *et al*. (1999) and Simón *et al*. (1999) for reviews of the soils of the area.

2.3. Sampling and Analysis

Eight sampling sites were selected along the Guadiamar floodplain, along a river stretch of about 40 km. Sites 1 and 2 were situated in locations not affected by the mine spill (unaffected sites). Site 1 was located near Gerena Bridge, upstream from the Aznalcóllar mine. Site 2 was located at 'Soberbina' farm, about 4.5 km from the mine, on an upper river terrace not affected by the spill. An additional sampling site, used as an external control (Site 3), was located in another river basin (Rivera de Huelva).

The affected sites were located in the area flooded by the spill. Site 4 was located at 'Soberbina' farm on a river terrace that was affected by the spill. Site 5 was at 'Doblas' bridge, 12 km from the mine. Site 6 was at 'Lagares' bridge, 15 km from the mine. Site 7 was at Aznalcázar Bridge, 25 km from the mine. Site 8 (clay loam) was at 'Vado del Quema', 31 km from the mine. Site 9 was at the 'La Tiesa' farm, 38 km from the mine, very close to the Guadalquivir marshes. Table I summarize the location and some characteristics of the sampling sites (for a general view of the area and the sampling sites see the special issue about the Aznalcóllar accident in Grimalt and Macpherson, 1999).

TABLE I

Texture, acidity and $CaCO_3$ concentration of the soils studied (0–25 cm, mean values ± standard error, $N = 3$)

Site	Longitude/latitude	pH	$CaCO_3$	Texture
Unaffected by the spill				
1	37°31′28″N, 6°11′18″ W	8.1 ± 0.2	1.6 ± 1.5	Loamy sand
2	37°27′28″N, 6°12′23″W	8.3 ± 0.1	20 ± 1.3	Clay loam
3	37°29′11″N, 6°01′32″W	8.3 ± 0.1	2.5 ± 1.0	Sandy loam
Spill-affected				
4	37°27′45″N, 6°12′55″W	6.9 ± 0.1	2.9 ± 2.2	Clay loam
5	37°24′58″N, 6°13′35″W	7.5 ± 0.3	12 ± 0.4	Clay loam
6	37°22′27″N, 6°13′42″W	8.1 ± 0.1	7.4 ± 0.7	Clay loam
7	37°18′11″N, 6°15′39″W	8.0 ± 0.1	7.1 ± 0.4	Silt loam
8	37°14′46″N, 6°15′53″W	7.2 ± 0.4	12 ± 4.7	Clay loam
9	37°14′44″N, 6°14′36″W	7.9 ± 0.2	4.9 ± 1.8	Loam

In each site, the aboveground part of the grass was sampled in three different patches separated by a distance of at least 30 m. Sampling was carried out in December 2001. Plant samples were washed (for approximately 10 s) with a solution of phosphate-free detergent (Extran MA03 Merck), then with a 0.1 N HCl solution and finally with distilled water. These washing techniques applied seemed effective for sample decontamination (Madejón *et al*., 2002). The plant material was then dried at 70 °C, ground and passed through a 500 μm stainless-steel sieve. A plant sub-sample was directly ground without previous decontamination by washing, to study the actual pollution of trace elements in the grass (including the adhered soil on the plant surfaces) and their possible toxic impact through consumption as forage.

Soil samples, at 0–25 cm depth, were taken from each patch where sampled grasses were growing. Soil samples were oven-dried at 40 °C and crushed to pass through a 2 mm sieve, and then ground to $<60\ \mu$m for S and total trace element determinations.

The plant material was analyzed for N by Kjeldahl digestion. Mineral nutrients (P, K, S, Ca and Mg), heavy metals and trace elements (As, Bi, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Tl and Zn) were extracted by wet oxidation with concentrated HNO_3 under pressure in a microwave digester. Analysis of the mineral nutrients Fe and Mn in the extracts thus obtained was performed by ICP-OES (inductively coupled plasma spectrophotometry). Analysis of the trace elements was performed by ICP-MS (inductively coupled plasma-mass spectroscopy). Unwashed plant samples were only analyzed by ICP-OES.

The accuracy of the analytical method was assessed by carrying out analyses of three BCR (Community Bureau of Reference) reference samples: BCR 62 (Olive leaves), CRM 279 (Sea lettuce) and CRM 281 (Ryegrass) (see Colinet *et al*., 1982; Griepink and Muntau, 1987, 1988, respectively). The values obtained for N Kjeldhal digestion, and for the elements determined by ICP-OES and by ICP-MS in the reference materials are shown in Table II. The indicative and certified values for the same materials are also shown.

Sulphur and trace elements (As, Bi, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn) in the soils were determined by ICP-OES, after digesting the samples ($<60\ \mu$m) with a mixture of conc. HNO_3 and conc. HCl (1:3 v/v, 'aqua regia') for two period of 9′ at a power of 600 and 350 W respectively in a microwave oven (Microwave Laboratory Station Mileston ETHOS 900, Milestone s.r.l., Sorisole, Italy); these values are referred to as "total" concentration. Available concentration of the trace elements (As, Cd, Cu, Fe, Mn, Ni, Pb and Zn) was determined by ICP-OES after extracting the samples (<2 mm) with a 0.05 M EDTA solution. Total and available concentrations of the trace elements are given on a dry weight basis.

To evaluate the soil pollution severity, we used the Pollution Load Index (PLI as defined by Tomlinson *et al*., 1980). This index is based on the values of the Concentration Factors (CF) of each metal in the soil. The CF is the quotient obtained by dividing the concentration of each metal in the soil ($C_{\text{heavy metal}}$) by the base line

TABLE II
Analysis of BCR reference samples (mean values ±95% confidence interval, $g\,kg^{-1}$ dry matter for macronutrient and $mg\,kg^{-1}$ dry matter for trace elements).

	CRM 279 (sea lettuce)		*CRM 281 (ryegrass)*		*BCR 62 (olive leaves)*	
Element	Certified	Experimental	Certified	Experimental	Certified	Experimental
N	(20.5 ± 0.04)	17.0 ± 0.07	(33.2 ± 0.05)	31.7 ± 0.13	–	
P	(1.80 ± 0.04)	1.50 ± 0.08	(2.30 ± 0.05)	2.15 ± 0.08	–	–
K	(13.9 ± 0.9)	13.0 ± 1.5	(35.2 ± 2.7)	31.6 ± 2.2	–	–
Ca	(27.4 ± 1.1)	30.8 ± 4.1	(7.20 ± 0.09)	6.75 ± 0.13	–	–
Mg	(14.4 ± 0.8)	14.1 ± 1.2	(1.66 ± 0.02)	1.59 ± 0.02	–	–
As	3.09 ± 0.20	2.69 ± 0.11	0.057 ± 0.004	0.118 ± 0.014	(0.20)	0.16 ± 0.016
Cd	0.274 ± 0.022	0.202 ± 0.007	0.120 ± 0.003	0.117 ± 0.005	0.10 ± 0.02	0.074 ± 0.009
Cu	13.14 ± 0.37	11.63 ± 0.73	9.65 ± 0.38	9.76 ± 0.09	46.6 ± 1.8	44.4 ± 2.5
Fe	(2300 ± 100)	2113 ± 72.3	–			
Mn	(2030 ± 31.5)	1758 ± 64.8	81.6 ± 2.6	76.7 ± 0.4	57.0 ± 2.4	54.9 ± 0.46
Ni	(15.9 ± 0.4)	13.1 ± 0.53	3.0 ± 0.17	2.58 ± 0.15		
Pb	13.48 ± 0.36	12.47 ± 1.09	2.38 ± 0.11	2.29 ± 0.07	25.0 ± 1.5	27.4 ± 1.32
Tl	(0.038 ± 0.005)	0.027 ± 0.005	–	–	(0.03)	0.028 ± 0.002
Zn	51.3 ± 1.2	52.18 ± 3.29	31.5 ± 1.4	32.7 ± 0.2	16.0 ± 0.7	19.1 ± 0.65

Values in parenthesis are indicative. Experimental values are calculated from $N = 6$ (sea lettuce) $N = 5$ (ryegrass) and $N = 5$ (olive leaves).

or background value (concentration in unpolluted soil, $C_{\text{background}}$)

$$CF_i = \frac{C_{\text{heavy metal}}}{C_{\text{background}}}$$

Background values were estimated from the mean concentrations of As, Cu, Ni, Pb and Zn for unaffected soils in the area (Cabrera *et al.*, 1999). For each sampling site, PLI may be calculated as the nth root of the product of the n CF:

$$\text{PLI} = \sqrt[n]{(CF_1 \times CF_2 \times \cdots \times CF_n}$$

Values of PLI close to 1 indicate heavy metal loads near the background level, while values above 1 indicate soil pollution (Cabrera *et al.*, 1999).

2.4. Statistical analysis

Analyses of variance (ANOVA), considering only one factor, i.e., soil pollution level, were performed for the concentration of each trace element in the grass and soils. A multiple comparison of mean values was determined by the Tukey test. A significance level of $P < 0.05$ was used throughout the study. Data normality was tested prior to analysis, and when necessary, variables were transformed logarithmically, and normality was then passed in all cases. To investigate the global pollution trends in the soils, the principal component analysis (PCA) was performed for the 'total' concentration of 11 trace elements in 27 soil samples. Previously, variables were transformed logarithmically to obtain a normal distribution.

Correlation analysis was performed between the concentration of trace elements in plants and their availability (after EDTA extraction) in the soil. The SPSS for Windows program was used for the statistical analyses mentioned above.

3. Results and Discussion

3.1. Soil residual pollution

In general, the spill-affected soils reached values clearly >1 for the pollution load indices (PLI), thus indicating a significant residual pollution level even after the soil clean-up operations (Table III). When considering jointly the PLI values and S concentrations (Table III), three different habitats were taken into consideration: i) unaffected sites (UN: sites 1, 2 and 3, as would have been the original conditions before the mine accident), ii) non-amended soils, still covered with sludge, (NA: sites 7, 8 and 9, near the river channel, more polluted as they were inaccessible to clean-up machinery), and iii) amended soils (A: sites 4, 5 and 6, as is the general situation in the affected area after removal of the sludge and the application of other soil restoration operations).

The 'total' concentration (extracted with *aqua regia*, also called 'quasi total' fraction) and available concentration (EDTA-extracted) values of trace elements in the soil samples are shown in Table IV. In general, the total concentrations

TABLE III
Total S (mean values ± SE, dry weight) and PLI values of the soils.

Site	S ($mg\,kg^{-1}$)	PLI (1)
Unaffected by the spill		
1	536 ± 327	0.49
2	290 ± 39.5	0.39
3	217 ± 42.5	0.51
Spill-affected		
4	7068 ± 3895	4.22
5	7690 ± 3356	4.77
6	1670 ± 355	1.43
7	3930 ± 1585	3.21
8	18250 ± 11854	6.69
9	4443 ± 2170	4.36

TABLE IV

Total and potentially available (EDTA extracted) concentrations (mg kg^{-1}) of iron and seven trace elements in soils (mean ±S.E.)

Element	Soil			Background values (Bowen, 1979)
	Non affected	Non-amended	Amended	
Total				
As	8.95 ± 1.1 b	246 ± 73.8 a	94.7 ± 19.6 ab	0.1–40
Bi	0.17 ± 0.03 b	3.49 ± 1.0 a	0.96 ± 0.2 b	0.1–13
Cd	1.00 ± 0.1 b	4.08 ± 0.45 a	2.94 ± 1.2 a	0.01–2
Co	10.1 ± 1.2 b	17.5 ± 1.5 a	13.2 ± 0.9 ab	0.05–65
Cr	33.4 ± 2.6 a	36.8 ± 3.8 a	30.6 ± 3.1 a	5–1500
Cu	16.8 ± 1.95 b	180 ± 19.1 a	129 ± 25.8 a	2–250
Fe	22700 ± 2215 b	43194 ± 4787 a	28500 ± 1725 b	2000–550.000
Mn	497 ± 48.6 a	609 ± 88.5 a	523 ± 29.2 a	20–10000
Ni	14.9 ± 1.6 b	25.2 ± 2.65 a	18.0 ± 1.0 b	2–750
Pb	10.0 ± 1.6 b	366 ± 130 b	161 ± 31.8 ab	2–300
Zn	47.6 ± 4.3 b	629 ± 96.2 a	435 ± 83.8 a	1–900
Available				
As	<0.1	0.35 ± 0.07	0.32 ± 0.07	
Cd	0.26 ± 0.13 b	1.08 ± 0.19 a	0.87 ± 0.17 a	
Cu	4.67 ± 0.63 b	39.0 ± 5.3 a	31.7 ± 7.6 a	
Fe	91.8 ± 18.0 ab	378 ± 245 a	62.0 ± 7.3 b	
Mn	76.4 ± 12.1 a	76.5 ± 6.5 a	66.3 ± 5.0 a	
Ni	0.49 ± 0.07 ab	0.68 ± 0.11 a	0.37 ± 0.05 b	
Pb	4.7 a ± 0.5 b	23.7 ± 6.4 a	18.1 ± 2.1 a	
Zn	3.5 ± 0.6 b	107 ± 18.3 a	63.6 ± 14.4 a	

For Bi, Co and Cr the EDTA values were not determined. Background (total) values according to Bowen (1979) are also indicated. For each element, values followed by the same letter do not differ significantly ($P < 0.05$).

were within the normal background values reported by Bowen (1979), with some exceptions. The total values of As, both in the NA and A soils, and total Cd in the NA soils, were greater than the upper limit of the range of background values; some values of Pb in the NA soils and Cd in the A soils were also greater than this limit.

The mean values of total trace elements in the soils followed the trend NA > A > UN. The relative increase of the total values in the NA soils by comparison with the UN soils (control) followed the trend Pb > As > Bi > Zn > Cu > Cd, while for A soils the trend was Pb > As > Zn > Cu > Bi > Cd. It should be emphasized that the biggest differences were found in the values of Pb: the mean values in the NA and A soils were 36.6 and 16.1 times greater respectively, than in the UN soils. The least differences were recorded for Cd: mean values of 4.1 and 2.9 times greater in the NA and A soils than in UN soils. After these observations it was possible to define the UN soils as 'non-polluted', the A soils as 'fairly polluted' and the NA soils as 'highly polluted'.

The total concentrations of Fe, Co, Ni and especially Cr and Mn in the NA and A soils experienced little or even null increases compared to the UN soils (however, the increases of Fe and Ni in the NA soils, compared to the A and UN soils, were significant for both the total and the EDTA-extracted fractions).

The values of the EDTA-extracted trace elements also increased in the NA and A soils in comparison with the UN soils. The extent of the increase was different for each element and followed the trend Zn > Cu > Pb > Cd > As in both the NA and A soils. The availability of a particular element in a soil does not always correspond to its 'total' concentration. In this study, the increases of Pb in the NA and A soils, compared to the UN soils, were greater for the 'total' fraction than for the available (EDTA-extracted) fraction.

Differences in trace element availability among soils seem not to be related to their pH, neutral or slightly alkaline (Table I) in all sampling sites (pH were practically the same in 1999 (first study) and 2001 (this study). Low pH increases metal availability by competing H^+ with the metal ions for negative charges on the colloids, thus releasing metals (Greger, 1999). This was not the case in this study, despite the slight decreases of pH recorded for NA soils. Differences of availability could thus be related to the greater concentration of sludge in the NA soils than in the A soils (greater extraction by EDTA) and amendment addition in the A soils.

The soils from the Guadiamar floodplain, which were affected by the spill, still remained polluted by trace elements 3 years after the accident. The clean-up and restoration operations (amendment addition), despite being absolutely necessary, only partially alleviated trace element pollution. In many cases, cleaning machinery operations buried part of the sludge (previously confined to the topsoil) and increased the concentration of metals. The result was a patchy and irregular distribution of the metal pollution along the Guadiamar and Agrio valleys.

The multivariate analysis (PCA) of the soil samples reflects the differential distribution of the trace elements. The first axis explained most of the variance (68.7%), and was determined by the concentration of six elements (having higher load factors): Cd, As, Cu, Zn, Pb and S. The distribution of soil samples along this axis reflects a main gradient of spill-induced pollution (Figure 1). Most of these elements are chalcophilic and were abundant in the sludge; their relative abundance in the polluted soils is inter-correlated.

The second axis reflected a secondary, less explicative gradient (15.5%), and was associated to Cr, and to a lesser extent, Mn. These lithophilic elements reflect a secondary chemical gradient within the soil samples, probably related to the geological nature of the parent material, and not to the spill pollution. A similar pollution pattern was found in a study of soils and biomonitoring role of poplar (*Populus alba*) in the Guadiamar river (Madejón *et al*., 2004).

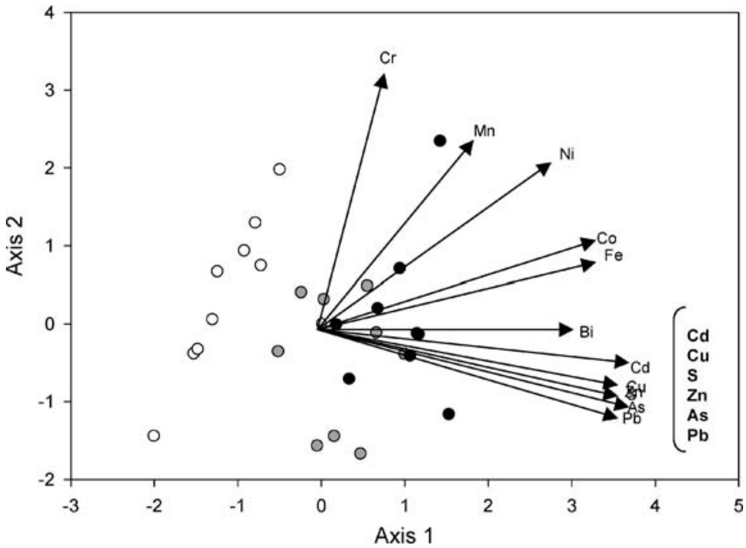

Figure 1. Ordination of soil samples by the two first axes of principal component analysis, according to their concentration of Fe and eleven trace elements. White symbols correspond to the unaffected sites, gray symbols to the amended sites, and black symbols to the non-amended sites. Vectors represent the loading factors (multiplied by five, for clarity) of the elements.

3.2. Effects of the residual pollution on plant macronutrients

Soil pollution caused significant changes in the concentration of macronutrients (N, K, Ca and Mg) in *Cynodon* (Table V). The plants growing in the NA soils had lower concentration values of N than in the UN soils, whereas concentration values of S, Ca and Mg were higher. The plants growing on the A soils showed no significant differences from UN soils in N (and P), but the concentration of K was lower, and the concentrations of S, Ca and Mg were higher. In general, the plants growing in the spill-affected soils tended to have lower concentrations of N, P and K and higher concentrations of S, Ca and Mg than in the unaffected soils.

Metals such as Cd can limit the N–NO_3, K, Ca and Mg uptake by plants, reducing their concentrations in plant tissues (Fodor *et al*., 1995; Boussana *et al*., 1999; Hagemeyer, 1999; Stolt and Oscarson, 2002). In the case of N, that uptake decrease

TABLE V
Main nutrient concentrations (%) in the *Cynodon* tops (mean values ± S.E., dry matter, $N = 9$)

Soil	N	P	K	S	Ca	Mg
Unaffected	1.84 ± 0.11 a	0.18 ± 0.02 a	1.83 ± 0.12 a	0.40 ± 0.01 b	0.43 ± 0.03 b	0.17 ± 0.02 b
Non-amended	1.26 ± 0.10 b	0.16 ± 0.01 a	1.64 ± 0.10 ab	0.57 ± 0.04 a	0.67 ± 0.03 a	0.21 ± 0.01 ab
Amended	1.70 ± 0.70 a	0.15 ± 0.01 a	1.24 ± 0.16 b	0.58 ± 0.02 a	0.65 ± 0.06 a	0.23 ± 0.01 a

Values followed by the same letter in the same column do not differ significantly ($P < 0.05$).

seems to be an indirect consequence of changes in the fluidity and H-ATPasa activity of the plasma membrane (Fodor *et al.*, 1995; Stolt and Oscarson, 2002). Also, microorganisms are reported to be sensitive to trace elements (e.g., Tl), and the inhibition of nitrate formation may have an impact on N uptake by plants (Kabata-Pendias and Pendias, 1992). The decrease in N uptake would be a constraint on the plant, taking into account that this element is often the limiting factor for *Cynodon*. N-fertilizers are routinely used in order to increase the forage and turf value of this species. In this context, the addition of organic matter to A soils, that can greatly enhance microorganism activity and non simbiotic N fixation (Cabrera *et al.*, 1996), could have contributed to the comparatively high N content in *Cynodon* growing thereon (Table V).

The increase of S in spill-affected plants was a consequence of the high S content in the sludge, whereas the increases of Ca, and to a lesser extent Mg, may respond to a periodical or occasional pyritic oxidation which could enhance $CaCO_3$ dissolution. That increase in Ca concentration could also induce a protective action against the toxicity of metals and metalloids (Carbonell *et al.*, 1998), being thus a positive feature for plant growth.

3.3. Effects of the residual soil pollution on trace elements in plants

The high pollution level of the NA soils induced a high concentration of trace elements in *Cynodon* (Figure 2); all the concentration values in the plant tissues, except those of Fe and Zn, were significantly greater in the NA soils than in the UN soils. On the contrary, except for Cd, in the A soils the plants did not show significant differences in trace element concentration when compared to the plants of UN soils.

The high concentration of As in the sludge, up to 4000 mg kg^{-1} (Cabrera *et al.*, 1999), caused some social alarm due to its known toxicity; however, the As in the plant tissues was far lower in all the soils than the maximum level (50 mg kg^{-1}) recommended for livestock forage (Figure 2; Table VI), and also lower than the phytotoxic range for most plants (3–10 mg kg^{-1}, Table VI). The As in plant tissues from the A soils were in the range considered normal for plants (Figure 2; Table VI). The As in unwashed plants (as would be ingested by animals; data not shown) were also lower than the limit for livestock: a mean value of about 5 mg kg^{-1} with a maximum value of 10 mg kg^{-1} in a sample from a NA soil.

The concentration values of As documented for 2001 were, in general, lower than those reported for 1999 (Madejón *et al.*, 2002); this seems to reflect a progressive amelioration of the soil conditions in the affected area derived from the restoration operations. In one of the most representative A soils (site 7 at Aznalcázar), the As in the plant tissues decreased from 6.08 mg kg^{-1} in 1999 to only 0.63 mg kg^{-1} in 2001. Thus, the risk of As toxicity for wild animals seems to have decreased in the spill-affected area after the partial soil restoration.

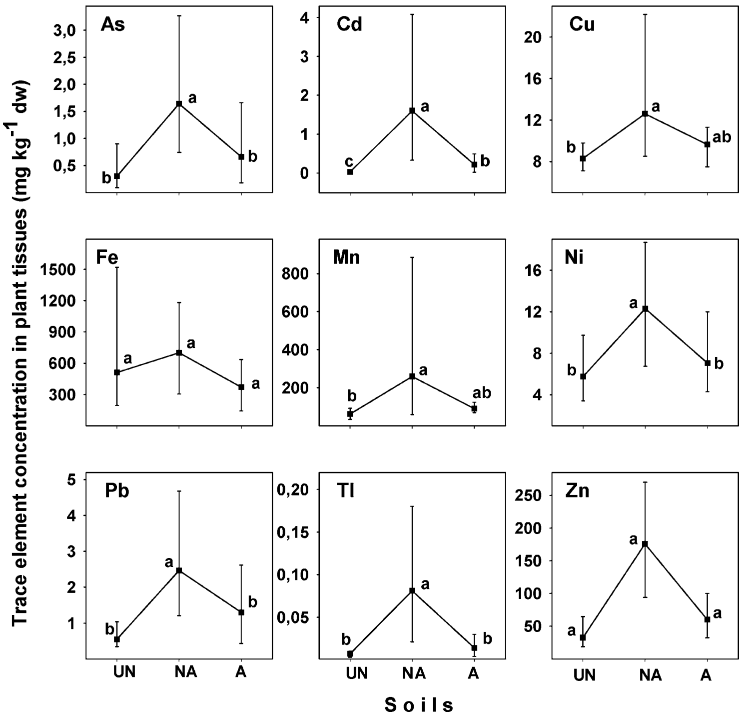

Figure 2. Trace element concentrations (mean values on dry matter) in plant tissues of *Cynodon dactylon* in the three soil types (unaffected soils, UN; non-amended soils, NA and amended soils, A; $N = 9$). The interval between maximum and minimum values is shown. Same letters indicate non significant difference between soil type, after ANOVA anlysis.

A temporal lowering of trace element concentrations in plant tissues is supposed to be based on a reduction of their availability in soils (amended with organic matter and Fe-oxides in our case). In the most representative A soil (site 7), the EDTA-As was ca. 2 mg kg^{-1} in 1999, and only 0.56 mg kg^{-1} in 2001 (a fourfold reduction). Also, the influence of the external soil contamination on the grass surface has decreased progressively with increasing soil stabilization and vegetation cover. In the unwashed plants from site 7, the As concentration was 20.9 mg kg^{-1} in 1999 and 3.8 mg kg^{-1} in 2001.

Soil pollution also induced a significantly greater Cd concentration in the *Cynodon* plants, compared with UN soil plants (Figure 2). However, the levels in plants from the A soils (0.22 mg kg^{-1}) were below the maximum tolerated by livestock (0.5 mg kg^{-1}; Table VI), which corroborates the aforementioned progressive diminution of the risk toxicity in the A soils. The EDTA-Cd in A site 7 was ca. 1 mg kg^{-1} in 1999 and 0.76 mg kg^{-1} in 2001.

Nevertheless, Cd in plants from the NA soils is still excessive for animals (Figure 2; Table VI); it was even greater than the Cd concentrations reported for

TABLE VI

Normal ranges in plants, phytotoxic concentrations and toxic levels for the livestock of some trace elements (from Chaney, 1989 and other authors, see footnotes)

Element	Normal levels ($mg\,kg^{-1}$ dry foliage)	Phytotoxic levels ($mg\,kg^{-1}$ dry foliage)	Maximum levels tolerated by livestock ($mg\,kg^{-1}$ dry diet)			
			Cattle	Sheep	Swine	Chicken
As inorg.	0.01–1	3–10	50	50	50	50
Cd	0.1–1	5–700	0.5	0.5	0.5	0.5
Cu	3–20	25–40	100	25	250	300
		10–70[a]			300–500[b]	
Fe^{2+}	30–300	400–1000[c]	1000	500	3000	1000
					5000[b]	
Mn	15–150	400–2000	1000	1000	400	2000
					1000[b]	
Ni	0.1–5	50–100	50	(50)	(100)	(300)
Pb	2–5	–	30	30	30	30
Zn	15–150	500–1500	500	300	1000	1000
				1000[b]	2000[b]	
Tl	0.05[d]	20[e]	–	–	–	–

Levels in parenthesis were estimated (by NRC) by extrapolating between animal species.
[a]Toxic levels for crops according to Gupta and Gupta (1998).
[b]Toxic levels according to Annenkov (1982).
[c]Critical toxicity concentration (Römheld and Marschner (1991).
[d]Typical concentration for a wide range of plants (Adriano, 2001).
[e]Excessive or toxic level for mature leaves of different plants (Kabata-Pendias and Pendias, 1992).

1999 samples from the affected soils (amended and sludge covered soils). These results support the legal measures taken to ban animal grazing in the spill affected area. But see Beyer (2000) who argues that Cd toxicity levels for wildlife have been exaggerated.

The affected soils remain polluted with Cu (Table IV) and the difference in concentration between plants from the NA and UN soils was significant (Figure 2); but, in general, Cu values in the plant tissues were within the normal range for plants and below the maximum tolerated by sheep, the livestock most sensitive to Cu (Table VI). This pattern of low accumulation of Cu in plants from the affected area has also been reported for other plant species: wild grasses (Madejón *et al*., 2002), sunflower (Madejón *et al*., 2003) and poplar (Madejón *et al*., 2004). Besides a comparatively high Cu retention in the soils, the presence of high concentrations of Fe and Zn could have inhibited Cu uptake by the plants (Kabata-Pendias and Pendias, 1992). Thus, Cu concentration in the plant tissues did not respond to the high content of the element in the sludge (up to 1550 $mg\,kg^{-1}$; Cabrera *et al*., 1999) and the affected soils (Table IV).

Unwashed samples from the NA soils occasionally reached values near 50 mg kg^{-1} of Cu, surpassing the toxic levels for sheep. This grass pollution figure supports the appropriateness of the urgent clean-up operations of the spill affected soils carried out in 1998, immediately after the mine disaster, and corroborates the need for the legal measures taken then, banning animal grazing in the area.

Iron in plant tissues has consistently decreased since 1999, following the general pattern followed by trace elements in *Cynodon*. In the plants from A site 7, the Fe concentration (434 mg kg^{-1}) in 2001 was far lower than the value of 1207 mg kg^{-1} recorded in 1999. Iron in plants from the A soils (about 400 mg kg^{-1}, Figure 2) was even somewhat less than that in the plants of the UN soils (control) and also less than the phytotoxic range for plants (Table VI).

The unwashed samples of the NA soils still remained polluted by external dust in 2001 (a maximum Fe concentration of ca. 3500 mg kg^{-1}), although to a lesser extent than the maximum values reported in 1999 (8323 mg kg^{-1}). In A site 7, the Fe concentration in the unwashed samples was 3510 mg kg^{-1} in 1999 and 1545 mg kg^{-1} in 2001. This temporal Fe reduction in the *Cynodon* tops also reflects the progressive decrease in external contamination caused by the clouds of dust generated during the soil clean up operations.

Manganese and Ni were not significant as polluting elements in the mine spill (Cabrera *et al.*, 1999). However, the Ni concentrations in the NA soils were significantly higher than those in the UN and A soils (Table IV). The differences in concentration of Mn and Ni in the plant tissues of the NA soils, compared to the plants from the UN and A soils (Figure 2) may respond in part to the aforementioned differences in concentration in the soils. However, the Mn and Ni concentrations were below the maximum tolerated by livestock, and also below the phytotoxic ranges for plants (Table VI). The maximum of 885 mg kg^{-1} recorded for Mn in plants from the NA soils (Figure 2) was excessive for swine, but not for other domestic animals (Table VI).

Lead was one of the metals with the highest concentration in the sludge (up to 9700 mg kg^{-1}, Cabrera *et al.*, 1999), and has been highlighted as one of the main sources of toxicity by the mine spill (Prat *et al.*, 1999). Lead poisoning is the most frequently diagnosed toxicological condition in veterinary medicine. However, despite the affected soils remain polluted by this element (Table IV), Pb in *Cynodon,* and the corresponding risk of toxicity for animals, has consistently decreased since 1999.

Lead in plant tissues of the A soils (ca. 1.5 mg kg^{-1}, a rather low value in plants, Table VI) was not significantly different from Pb in plants from the UN soils (Figure 2). Lead in plants from the NA soils was also low and in the normal range for plants (Figure 2, Table VI). In general, Pb values in the plant tissues were very much lower than those reported in 1999. In A site 7, the Pb concentration in *Cynodon* decreased from 12.1 mg kg^{-1} in 1999 to only 1.36 mg kg^{-1} in 2001.

The organic matter added to must have reduced trace element availability in the soils, Pb included; in A site 7 EDTA-Pb was ca. 30 mg kg^{-1} in 1999 and 22 mg kg^{-1} in 2001. Additionally, most Pb uptaken by plants remains in the root system (Adriano, 2001); thus, the soil-plant barrier (Chaney, 1989) may act against Pb toxicity, protecting the food chain.

The external concentration of Pb on the grass surface was reduced by soil stabilization and plant growth and renewal. Lead in unwashed plants from A site 7 was ca. 40 mg kg^{-1} in 1999, greater than the maximum of 30 mg kg^{-1} tolerated by livestock (Table VI). While Pb in plants from the same sites decreased down to ca. 15 mg kg^{-1} in 2001. However, Pb in some plant samples of the NA soils is still near the toxic levels; thus, the restraint on grazing in the area must be maintained and monitorization of the soil-plant system continued.

Despite a noticeable Tl content in the sludge (up to 60 mg kg^{-1}, Cabrera *et al.*, 1999), its concentration in *Cynodon* was low, partly explained by the very scarce mobility of this element in the sludge and the affected soils (Vidal *et al.*, 1999); in contrast to general data in literature (Kabata-Pendias and Pendias, 1992). Thus, Tl in plants from A site 7 in 2001, about 0.01 mg kg^{-1}, was lower than in 1999, 0.08 mg kg^{-1}.

The maximum Tl concentration recorded in plants from the NA soils, 0.18 mg kg^{-1}, was much lower than the excessive or toxic level of 20 mg kg^{-1} reported by Kabata-Pendias and Pendias (1992) for mature leaves of different plants. Only some accumulator plants (such as cultivated *Brassica* ssp.) have been documented as having a comparatively high Tl uptake in the Guadiamar basin (Soriano and Fereres, 2003). We also recorded a high Tl concentration (ca. 50 mg kg^{-1}) in the flowers of *Hirschfeldia incana* (L.) Lagr. Foss., a wild crucifer abundantly growing in heavily polluted soils in the Guadiamar basin, (Madejón *et al.*, in press). These flowers may be consumed by primary consumers, and Tl accumulation should be examined because of the possible impact on the trophic web. An extremely high accumulation of Tl in flowers was reported for *Gallium* sp. (17,000 mg kg^{-1} on a dry ash weight basis; Kabata-Pendias and Pendias, 1992).

The Zn concentrations in plant tissues followed the general trend recorded for most trace elements. In A site 7, the mean value of Zn, 65 mg kg^{-1} (Figure 2), was in the normal range for plants (Table VI) and lower than in 1999 (100 mg kg^{-1}). However, in the unwashed samples of the NA soils a maximum of Zn as high as 400 mg kg^{-1} was recorded (somewhat excessive for sheep, Table VI); a toxic value not detected in 1999. This fact reinforces the need for a periodical monitorization of the soil-plant system in the affected area, despite the general downward trend followed by the trace elements.

The concentrations of other trace elements, such as Co and Cr, were about twice (Cr) or three times (Co) greater in the plant tissues of the NA soils than in plants of the A and UN soils. The differences were significant for Co (0.6 mg kg^{-1} in the NA soils; 0.2 mg kg^{-1} in the A and UN soils) and Cr (4 mg kg^{-1} in the NA soils; 2 mg kg^{-1} in the A and UN soils). In the case of Bi, the difference was not significant. These three elements were lower or in the range considered normal for plants: 0.06 mg kg^{-1} for Bi; 0.01–4.6 mg kg^{-1} for Co and 0.016–14 mg kg^{-1} for Cr (Bowen, 1979). Also, the concentrations of Co and Cr were comparatively low in the sludge (Cabrera *et al*., 1999).

3.4. Using *Cynodon* for biomonitorization and phytoremediation

This species plays a role in soil stabilization, after the restoration practices done in the spill-affected area. Additionally, the plant may play a role as a biomonitor of the pollution level caused by trace elements such as As, Cd, Cu, Pb and Zn (Figure 3). Biomonitors are defined as organisms that contain information on the quantitative aspects of the quality of the environment (Markert *et al*., 2003). The accumulation of a trace element in a particular organ of a plant confirms its availability in the soil; as distinct from those situations having a high total metal concentration, but being immobile in the soil complex. Many plants concentrate trace elements in their aerial parts to levels many times higher than that in soil solution (Baker *et al*., 2000; Ma *et al*., 2001). Thus, biomonitors can be used to detect low concentrations that are not always easy to measure directly using chemical extraction techniques; even if they are measurable as total levels, their ecological relevance is often difficult to determine from soil concentrations.

The best correlations between the concentrations in plant tissues (tops) and their availability (EDTA extractable) in the soil were obtained for the chalcophile elements As, Cu and Zn. The siderophyle elements Fe and Ni, and the lithophyle Mn did not show any significant correlation (Figure 3). These elements also showed a poor or null correlation in the case of poplar leaves and stems sampled in the Guadiamar river (Madejón *et al*., 2004).

The plant-soil correlation coefficients obtained in 2001 were smaller than those for 1999: 0.654 versus 0.839 for As, 0.549 versus 0.923 for Cd, 0.528 versus 0.627 for Pb, and 0.693 versus 0.845 for Zn (see Figure 3 and Madejón *et al*., 2002). The correlation for Cu was not significant in 1999. These weaker correlations can be explained by the plants accumulating fewer amounts of trace elements in their tissues after the soils had amended and stabilized, and in general by the lower pollution range in both plant and soil.

A lower accumulation of toxic trace elements in the plant tissues is a relevant factor in favor of its role as a soil stabilizer species. 'Normal' concentrations of trace elements in plant tissues reduce the risk of metal dispersal, and the species is then suitable for phytostabilization. On the contrary, possibilities for phytoextraction using *Cynodon* are negligible, according to both the low biomass production (in the range of about 100–800 g dw m^{-2} in the affected soils) and the slow rate of trace element extraction.

For phytoextraction, plants should remove sufficient amounts of metals from the soil and concentrate them in their harvestable fraction. For this purpose, a very fast rate of growth and biomass production would be beneficial characteristics

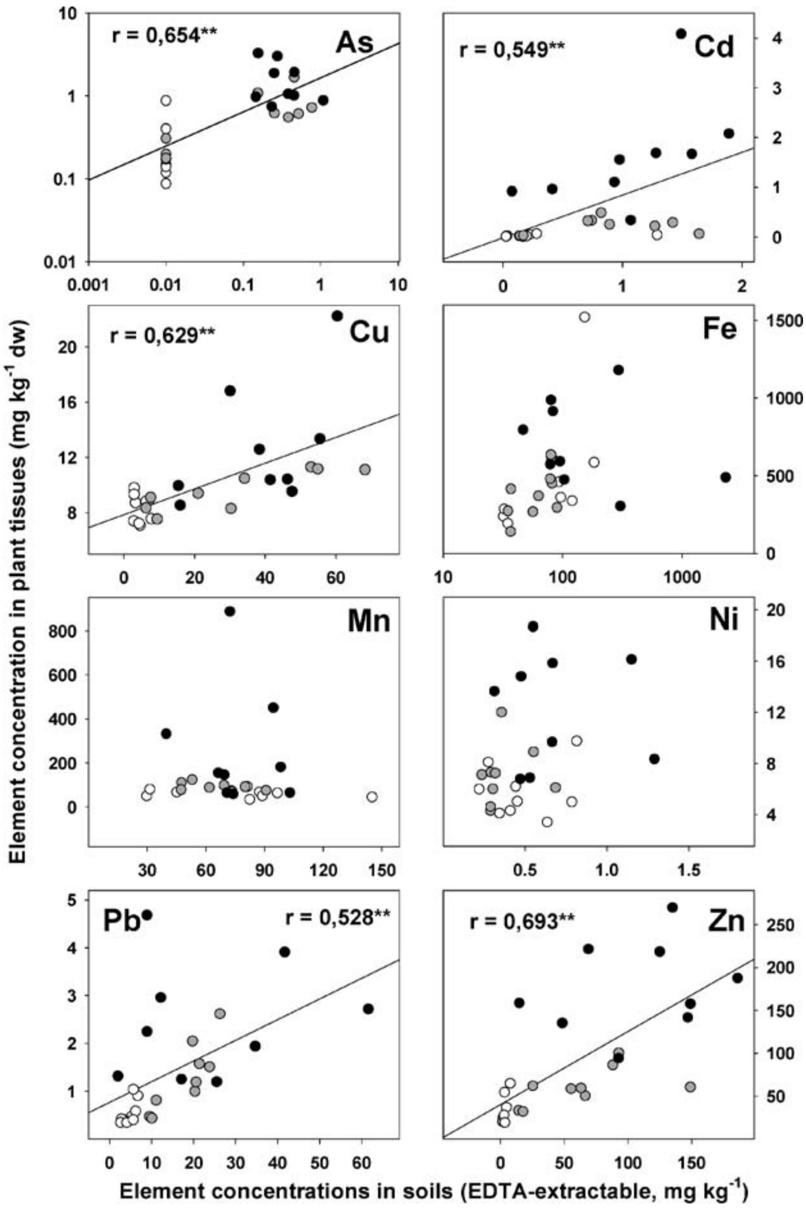

Figure 3. Correlation analysis between the availability of Fe and trace elements in soil (EDTA-extractable) and their concentration in plant tissues (*Cynodon* tops). White symbols correspond to the unaffected sites, gray symbols to the amended sites, and black symbols to the non-amended sites. In the case of As, the scale is logarithmic. When they are significantly correlated, the regression line has been drawn. Significance level indicated by ** means $P < 0.01$.

TABLE VII
Extraction of As, Cd, Pb and Zn (g ha^{-1}) by the above-ground part of *Cynodon* in different soils (the number of harvests needed to extract trace elements loaded by the spill in parenthesis)

Soil	As	Cd	Pb	Zn
Amended	2.56 ± 1.36 (245300)	0.88 ± 0.42 (6600)	4.83 ± 2.29 (170000)	209 ± 80.5 (7100)
Non-amended	5.67 ± 0.93 (155300)	5.77 ± 1.07 (1600)	8.78 ± 1.43 (144000)	671 ± 172 (3600)

(see review in Robinson *et al.*, 2003). However, there can be some difficulties for successful applying phytoextraction to contaminated soils. Among other reasons, this technique may require an excessive amount of time to reach safe levels, and most plant species well adapted to each particular scenario are not known to accumulate all the most environmentally toxic metals (and metalloids) such as Pb, Cd or As (Mertens *et al.*, 2004).

In this study, the extraction capacity of *Cynodon* for As, Cd, Pb and Zn in both A and NA soils was quite low (Table VII). For example, up to 245,300 years harvesting bermudagrass should be needed to decontaminate from As the amended soils in the spill-affected area of Guadiamar valley.

4. Conclusions

Three years after the Aznalcóllar mine accident, the concentration of trace elements measured in soil samples still indicates a noticeable residual pollution level. But in general, partial soil restoration and stabilization attained after the accident seem to have progressively reduced trace element availability in the soils and further accumulation in the tissues of *Cynodon*. Soil stabilization and plant renewal have also caused less external contamination of the *Cynodon* tops with trace elements (caused by the adherent soil).

Trace element concentrations in the plants were lower, in general, than those values reported in the same sites during 1999 (18 months after the accident), for both washed (plant tissues) and unwashed plants (as consumed by herbivores). However, there are still ‘highly polluted’ soils (practically inaccessible to the clean-up operations) where the unwashed tops of *Cynodon* reach concentrations of Cd, Fe, Mn, Zn and Pb, which may be excessive for different animals. The Cd level was excessive even in the washed tops (plant tissues). Thus, the restraint on animal grazing in the affected area must be maintained as long as possible and the monitorization of the soil-plant system continued, especially in the case of Cd.

This grass species seems to be suitable as a soil stabilizer for the spill affected soils, and also as a biomonitor for soil pollution by some chalcophile trace elements, such as As, Cu and Zn (and to a lesser extent Cd and Pb). However, its potential for decontamination of spill-affected soils by phytoextraction is negligible.

Acknowledgments

The PICOVER program from the Regional Ministry of Environment ("Consejería de Medio Ambiente, Junta de Andalucía"), Spain, supported this study. We thank José María Alegre for his help at different stages of the study. Trace element concentrations in plants were determined by Olga Cazalla at the Centre for Scientific Instrumentation, University of Granada.

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