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## Soundness of *in situ* lipid biomarker analysis: Early effect of heavy metals on leaf fatty acid composition of *Lactuca serriola*

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### Abstract

In previous studies we proposed the plant leaf fatty acid composition, and more precisely the ratio C18:3/(C18:0 + C18:1 + C18:2) as a tool to diagnose soil contamination *ex situ*. These studies led us to normalize this parameter as a biomarker of soil quality using *Lactuca sativa* growing under controlled conditions as the plant model species. The evaluation of the toxic effects of pollutants is more difficult to grasp in a real and more complex environment. Hence we developed a study to test the hypothesis that plant leaf fatty acid composition could be used also as a reliable tool to diagnose soil contamination *in situ*. We tested whether *Lactuca serriola* (prickly lettuce) could be used as a plant model species for such studies, primarily because it is widely distributed in various European countries and recorded at different habitats, and regarded as an ancestor of the cultivated lettuce *L. sativa* that was used for *ex situ* experiments. The results show that the lipid biomarker values were significantly lower in leaves of *L. serriola* grown on a metallurgic landfill soil than in leaves of plants harvested on surrounding countryside areas. To determine whether the value of the lipid biomarker in *L. serriola* leaves could be correlated to the soil metal content, we also measured the metal content of the soil corresponding to the rhizospheric areas from where the prickly lettuce leaves were harvested. The results of the Principal Component Analysis (PCA) show that the lipid biomarker is negatively correlated with both Ni and Cr content. Moreover, these results show that the lipid biomarker values measured *in situ* were repeatable from year to year, confirm that this biomarker measured *in situ* would provide an early indication of a plant's exposure to metal, and could be therefore used to facilitate or strengthen the diagnosis of soil contamination.

### Keywords:

*Lactuca serriola*; Lipid biomarker; Fatty acids; Metallurgical landfill; Chromium; Nickel

### I. Introduction

Soil is at the interface between geosphere, hydrosphere and biosphere and fulfils multiple environmental (*e.g.* storage, filtering and transformation), economic (*e.g.* source of raw materials, food production and biomass), social and cultural (*e.g.* platform of human activity, landscape support, cultural heritage) functions. Soil is also an ecosystem in its own right involving a huge quantity and variety of living organisms. As a result, soil appears as an essential resource for human societies and ecosystems. Hence, anthropogenic activities of various origins (agricultural, urban and industrial) that cause severe impacts on the health and/or the functioning of ecosystems have become a worrying problem. To enable the sustainable development of societies, it is now necessary to ensure soil protection. To initiate,

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follow, and ensure appropriate protective actions and management of soils, it is advisable to develop indicators to identify and quantify the disturbances and changes in soil quality, and to further determine the impacts on ecosystems. Currently, characterization and evaluation of soil pollution is mainly based on physicochemical analysis. However, to evaluate global environmental stress (e.g. chemical pollution, physical condition of soil, biological changes, etc.), biological indicators are necessary in complement to physicochemical analysis (Commission to the Council, the European Parliament, 2006). Among these biological indicators, biomarkers must be developed and used. A biomarker is defined as an observable and/or measurable change in an organism at a molecular, biochemical, cellular, physiological, or behavioral level, related to its exposure to at least one chemical pollutant (Lagadic *et al.*, 1997). These biomarkers must be able to detect early effects that may eventually become irreversible damage on the individual, community or ecosystem.

We have previously developed a biomarker of exposure, which have been used successfully for assessing the ecotoxicity of complex soils (Verdoni *et al.*, 2001, Le Guédard *et al.*, 2008 and [Le Guédard and Bessoule, 2011]). We showed that the leaf fatty acid composition of tomato or lettuce was modified when plants were grown on metal contaminated soil in a phytotron chamber. More precisely, it was shown that the C18:3/(C18:0 + C18:1 + C18:2) ratio (called “lipid biomarker”) decreases.

Although the results of numerous tests give proof that the lipid biomarker is a consistent and powerful tool to measure soil quality *ex situ* (Le Guédard and Bessoule, 2011), one potential limitation to the data is the controlled conditions under which the previous studies were carried out. In a real environment, the complex mixtures of pollutants, combined with physical alterations of habitat and climatic conditions can render experimental evaluation of the toxic effects of pollutants more difficult. Therefore, one of our aims was to test the hypothesis that the lipid biomarker could be also successfully used *in situ* to diagnose soil contamination.

The site investigated in the framework of the “Bioindicator” programme funded by ADEME (French Environment and Energy Management Agency) was a highly polluted metallurgical landfill. Among the plant species growing in this site, *Lactuca serriola* (prickly lettuce) was chosen as the plant model species because the edible lettuce (*Lactuca sativa*) was used successfully in our previous studies carried out *ex situ* (Le Guédard *et al.*, 2008 and Le Guédard and Bessoule, 2011). In the present paper, the leaf fatty acid composition of 20 *L. serriola* plants harvested on the metal-contaminated landfill was compared to the fatty acid composition of 20 prickly lettuce leaves harvested on areas located in the vicinity of the landfill. Then, to determine whether the value of the lipid biomarker in *L. serriola* leaves could be correlated to the soil metal content, the fatty acid composition of 27 prickly lettuce leaves harvested on the metal-contaminated landfill, was compared to the metal content of the rhizospheric soils where the harvested prickly lettuce were growing.

## II. Material and methods

### II.1. Biological features of *L. serriola*

*L. serriola* is a winter annual or biennial plant of the Asteraceae family whose distribution area extends mainly in temperate Eurasia. This species is a common weed present throughout France, colonizer of disturbed habitats like roadsides, vacant lots, slopes and other dry places. *L. serriola* is regarded as a progenitor and part of the primary gene pool of cultivated lettuce, *L. sativa* L. (Lebeda *et al.*, 2001 and Oregon State University, 1998). This plant has strong self-fertilizing ability (Lebeda *et al.*, 2001 and Mejías, 1993) and is primarily pollinated by insects. It flowers from July until September. Regarding lipid metabolism, as other asteraceae (Mongrand *et al.*, 1998), *L. serriola* is a C18:3 plant. In other words, *L. serriola* leaves do not contain 16:3 fatty acid (on control soil, they contained: 20.25 ± 1.97% of 16:0, 2.27 ± 0.38% of 16:1, 1.57 ± 0.22% of 18:0, 1.91 ± 0.31% of 18:1, 9.74 ± 1.96% of 18:2 and 64.27 ± 1.97% of 18:3 fatty acids (n = 20)).

## II.2. Site description

*L. serriola* leaves were harvested on a highly polluted metallurgical landfill located near Lyon (Rhône, France), behind an iron and steel factory which was still in activity. The landfill covers about 15 ha and has been used since 1850 to deposit iron and steel foundry wastes. As a result, the soil of this site is not native from the underlying bedrock, but is a metalliferous soil. Because the site was still in activity, vegetation cover with both trees and herbaceous species on this site was very heterogeneous due to engine traffic and new waste deposits. The site, located about an altitude of 250 m, is under a temperate continental climate with mean annual temperature of 10 °C, including minimal and maximal values ranging from -26 °C to +41 °C. The annual precipitation average is 750 mm.

## II.3. Soil analyses

Soil samples collected were dried at 60 °C for 48 h, sieved to 2 mm and ground to pass a 250- $\mu$ m mesh sieve before analyses. The pH value of the soil was determined in deionised water using a 1:2 soil:water ratio according to the NF ISO 10390 procedure (AFNOR, 1999). The total Na<sup>+</sup> content was determined using cobaltihexamine chloride. Results showed a homogenous Na<sup>+</sup> content on various areas of the metallurgical landfill soil, and evidenced a low amount of Na<sup>+</sup>:  $0.0207 \pm 0.005$  mol/kg, which represented less than 0.1% (w/w) of NaCl in this soil. Concentrations of heavy metals in various extracts were performed by ICP-OES, after aqua regia extraction as previously described (AFNOR, 1994 and Remon *et al.*, 2005). Limits of detection were 0.06, 0.14, 1.17, 2.05, 2.38 and 15.51  $\mu$ g l<sup>-1</sup> for Zn, Cd, Cr, Cu, Pb and Ni, respectively. To check the analytical precision, randomly chosen samples (about 20% of the total number) were measured in triplicate. The relative standard deviation was routinely between 1 and 8%, and never higher than 10%. For quality control of soil analyses for total metals, certified reference soils NCS DC 73323 and 73006 from the China National Analysis Center for Iron and Steel were included in the batch of samples submitted to aqua regia extraction. Average recoveries (n = 6) were 83, 73, 76, 85, 104 and 99% for Zn, Cd, Cr, Cu, Pb and Ni, respectively.

## II.4. Fatty acid extraction, analysis and identification

About 10–100 mg of fresh tissue were placed in screw-capped tubes containing 1 ml of methanol, 2.5% H<sub>2</sub>SO<sub>4</sub> (v/v). After 1 h of heating at 80 °C, 1.5 ml of H<sub>2</sub>O and 0.75 ml of hexane were added. Fatty acid methyl esters (FAMES) were extracted into hexane by vigorous shaking, after a two-phase system was established by centrifugation (1500  $\times$  g, 5 min). The methyl esters were analysed by gas chromatography (Hewlett-Packard 5890 series II, Agilent, Waldbronn, Germany) on a 15 m  $\times$  0.53 mm Carbowax column (Altech, Deerfield, IL, USA) with flame ionization detection and helium as carrier gas. The temperature program was as follows: the initial temperature of 160 was held for 1 min, followed by a 20 °C/min ramp to 190 °C and a second ramp of 5 °C/min to 210 °C, and maintained at this temperature for a further 6 min (total run time 13 min) (Le Guédard *et al.*, 2008). Routinely, fatty acids were identified by comparing their retention times with standards (Sigma Chemical, St. Louis, MO, USA). To analyse data in the same way as previous *ex situ* experiments (Le Guédard *et al.*, 2008 and Le Guédard and Bessoule, 2011), results are expressed as the C18:3/(C18:0 + C18:1 + C18:2) ratio.

## II.5. Statistical analysis

Statistical treatments were performed with the software XLSTAT. The Shapiro–Wilk test was used to check normal distribution. When the variables follow a normal distribution, parametric statistical tests (Student's t-test, Pearson's correlation) were used, whereas non-parametric statistical test (Spearman's rank correlation coefficient) was used when the distribution of the variables was not normal. Pearson's correlations were used to test the associations between the plant size and the lipid biomarker values measured on the leaves from lettuce harvested on the surrounding countryside or on the metallurgical landfill. Lipid biomarker and metal content values were analysed by Principal Component Analysis (PCA) from covariance matrix.

### III. Results

#### III.1. Lipid biomarker of *L. serriola* harvested on the metallurgical site or on the surrounding countryside areas

The lipid biomarker was successfully used *ex situ* for assessing the ecotoxicity of complex soils (Verdoni *et al.*, 2001 and Le Guédard *et al.*, 2008). In order to determine whether it could also be a powerful tool *in situ*, a first set of analyses was carried out with *L. serriola* plants harvested both on a contaminated site and on areas located in the surrounding countryside of this site (control area). The contaminated site under study is a highly metallurgical landfill that contains high levels of Cr, Zn, Ni and Pb (concentrations ranging from 601 to 1393 mg kg<sup>-1</sup>) (Remon, 2006). The metal content in this soil is therefore greatly higher than the content observed in the soil of the control area (Table 1). The leaf fatty acid compositions of 40 prickly lettuces found on this metallurgical site (20 plants) and on the surrounding countryside areas (20 plants) were determined. Figure 1 shows the distribution of C18:3/(C18:0 + C18:1 + C18:2) ratio values for plants harvested both on the metallurgical landfill and on the surrounding countryside areas. The mean of C18:3/(C18:0 + C18:1 + C18:2) ratios (usually used *ex situ* as a “lipid biomarker”) was significantly ( $p = 0.008$ ; Student's t-test) lower in leaves of *L. serriola* grown on the metallurgical landfill ( $4.10 \pm 1.08$ ) than when grown on the surrounding countryside areas ( $5.02 \pm 1.00$ ). These results are in agreement with those previously obtained *ex situ* with *L. sativa* (Le Guédard *et al.*, 2008 and Le Guédard and Bessoule, 2011) and highlight an adverse effect of wasteland soil on plants.

Plants under analysis had highly variable size, ranging from 0.1 to 1.2 m. To determine whether lipid biomarker values were correlated to plant size, a Pearson correlation analysis test was carried out (Figure 2). Result clearly showed that, whatever the soil on which lettuces grown, there was no correlation between the lipid biomarker values and the plant size (surrounding countryside:  $R = 0.392$ ,  $p = 0.087$ ; metallurgical landfill:  $R = -0.077$ ,  $p = 0.755$ ). Similarly, the lipid biomarker values were independent of the vegetative coverage of the harvesting location, the level of sun exposure or the stage of development of the harvested plant (vegetative, pre-bloom, bloom) (data not shown). However, the lipid biomarker seemed to vary more widely according to the areas sampled within the contaminated site. Significantly higher values of 5.32, 5.31 and 6.09 were obtained for three plants harvested at the border of the site, and it was possible that this area was less contaminated than the other areas sampled further into the contaminated site. Such an assumption was further investigated.

#### III.2. Correlation between the lipid biomarker measured with *L. serriola* and the metal content in soils

Three leaves per *L. serriola* plant were sampled again one year later (also mid of June) from each of nine areas of the metallurgical site. The analysis of fatty acid composition showed high repeatability of the measurements since the mean of the 27 lipid biomarker values was  $4.18 \pm 0.84$ , closed to the values ( $4.10 \pm 1.08$ ) determined a year ago on the same site. In addition, as observed after the first sampling campaign, the lipid biomarker values (5.61, 5.86 and 5.37) were higher on an area located at the site border, than on the other landfill areas.

To determine whether the lipid biomarker values were correlated to the soil metal contents, soil samples corresponding to the various rhizospheric areas of plants were collected simultaneously to the lettuce leaf harvest. Measurements of the total metal content in soils of the metallurgical site (Table 2) showed that Cr, Zn, Ni, and Cu were the most abundant heavy metals in the soil. These metals were present at a concentration range from 504 to 1346 mg kg<sup>-1</sup> as observed in previous studies from the same site (Remon, 2006). This indicates that the average metal content on this site did not greatly vary as a function of time. Moreover, the performed chemical analysis confirmed that the area located on the border site and displaying the highest lipid biomarker values (L4) was the area containing the lowest metal amounts. A statistical analysis by PCA was further carried out. Result (Table 3) shows that the lipid biomarker is significantly ( $p < 0.05$ ; Spearman's rank correlation coefficient) negatively correlated with Ni and Cr, but did not correlate with other metal (Cd, Cu, Pb and Zn) contents. In addition, a significant positive correlation is observed between Cr and Ni. The correlation

circle of the two first principal components analysis is shown in Figure 3. The first two principal components account for 83.46% of the total variation (eigenvalue > 1). Ni and Cr are correlated with the first component (representing 46.27% of the total variation). These variables are positively correlated with the first principal component. The second component (37.19% of the total variation) is mainly linked to Cd, Pb and Zn with a positive correlation. Thus, in the correlation circle, Ni and Cr form a cluster of correlated variables, whereas Zn, Pb and Cd taking a more independent position. As regards the lipid biomarker, is negatively correlated with the first principal component, and as a consequence with Cr and Ni. Thus, all together, these results clearly indicate a relationship between low (resp. high) lipid biomarker values and high (resp. low) Cr and Ni content. Nevertheless, since Cr and Ni contents were highly correlated, it is not possible to determine which one of these metals (or both of them) impacted the fatty acid composition of *L. serriola* leaves.

#### IV. Discussion

The aim of this study was to determine whether the fatty acid composition of *L. Serriola* leaves could be used successfully as a biomarker of soil contamination *in situ*. We showed that *L. serriola* grown on a contaminated landfill displayed lower lipid biomarker values than when grown on the areas located in the vicinity of the site. This control area was located at 1–2 km from the metallurgic landfill and at the same altitude (*i.e.* in the same geographical area), and climate conditions were the same in both of these contexts. Nevertheless, it cannot be excluded that a difference in a parameter other than the metal content between the countryside and metallurgical landfill soils was responsible for the decrease in the biomarker values. For this reason, we further determined whether within the same location (*i.e.* on the same soil), the biomarker values were correlated to the metal content of the rhizospheric areas. These analyses highlighted a clear relationship between lipid biomarker values and (some) metal content in the soil of the various areas of the metallurgic landfill. Hence, it appeared that the results obtained with *L. serriola in situ* were in perfect accordance with those previously obtained with *L. sativa* grown in a phytotron chamber (Le Guédard *et al.*, 2008). Actually, results obtained *ex situ* with *S. lycopersicum* and *L. sativa* showed that the decrease in lipid biomarker values mainly resulted from a decrease in the percentage of linolenic acid (C18:3) (Verdoni *et al.*, 2001 and Le Guédard *et al.*, 2008). Such a decrease in the amount of tri-unsaturated fatty acids in higher plants was also observed for tomato seedlings grown in culture solution containing copper or cadmium (Ouariti *et al.*, 1997 and Djebali *et al.*, 2005), as well as for pepper and rape seedlings grown in nutrient solution supplemented with Cd (Jemal *et al.*, 2000 and Ben Youssef *et al.*, 2005). Interestingly, Cu and Pb did not induce change in the fatty acid composition of cultured lichens that do not contain tri (or more) unsaturated fatty acids (Guschina and Harwood, 2006), whereas *Rhytidiadelphus squarrosus*, a moss that contains high levels of polyunsaturated fatty acids (PUFAs), when obtained from a Pb-polluted site has significantly less PUFAs containing three or more double bonds (Guschina and Harwood, 2002). All together, these results highlight the fact that fatty acids containing at least three double bonds are more specifically targeted by heavy metal contamination. In agreement, the decrease in lipid biomarker values in leaves of *L. serriola* grown *in situ* on contaminated soils resulted from a decrease in the percentage of linolenic acid (C18:3):  $64.3 \pm 2.0\%$  ( $n = 20$ ) and  $58.1 \pm 3.4\%$  ( $n=20$ ) on the surrounding countryside areas and on the landfill soil, respectively. In higher plants, tri-unsaturated fatty acids are mainly associated with chloroplast lipids, and more precisely with thylakoid membrane lipids. Indeed, the tri-unsaturated fatty acids content in photosynthetic membranes is crucial to various parameters such as on chloroplast ultrastructure, thylakoid membrane protein and chlorophyll content, electron transport rates, and the thermal stability of the photosynthetic membranes (Hugly *et al.*, 1989). In this study, it is shown that the decrease in the lipid biomarker on contaminated soil is not related to the size of the lettuce or the level of sun exposure or the stage of development. Thus, since photosynthesis provides the material necessary for plant growth and development, our results suggest that metals in soil could initially induce an early alteration on chloroplast membranes, which could lead later to the disruption of photosynthetic function and as a result

inhibit plant growth. In other words, these results show that the lipid biomarker measure an early effect of contaminants that has not yet had an impact on plant physiology such as plant growth.

To cause toxic effects, metals ions must be transferred from soils to plants. However, this transfer depend on many parameters including the heavy metal mobility, which depends on factors (Violante *et al.*, 2010) such as the type of metals, their concentration in the soil and their bioavailability, modulated by the presence of organic matter, pH, redox potential, concentration of other elements. Once in the plant, the impact of metal-ion toxicity on biochemical processes depends on the plant species, the time exposure, and metal concentration in the plant (Sanità di Toppi and Gabbrielli, 1999). The contaminated site under study was a landfill that contains high levels of Cu, Ni, Zn and Cr (504, 582, 1091 and 1346 mg kg<sup>-1</sup>, respectively) that were largely attributable to industrial activities. Among these metals, the decrease in the lipid biomarker values *in situ* is positively solely correlated with Cr and Ni contents. Thus, these results suggest that (i) Cr and Ni are the only bioavailable metals in the contaminated soil, (ii) these metals are the only ones which induce change in the leaf fatty acid composition of *L. serriola*, or (iii) these metals are in competition with other metals in soil, and thus may inhibit their absorption. Cr does not play any role in plant metabolism, whereas Ni is an essential micronutrient for normal plant growth and development (Brown *et al.*, 1987). Nevertheless, at high concentrations both Ni and Cr have various adverse effects such as inhibition of growth and development, induction of leaf chlorosis, necrosis and wilting and disruption of photosynthesis in various plant species (Sun and Wu, 1998, Gajewska *et al.*, 2006, Tripathy *et al.*, 1981, Boominathan and Doran, 2002, Boisvert *et al.*, 2007, Van Assche and Clijsters, 1983 and Dixit *et al.*, 2002). In our case, the absence of a difference in plant size between the contexts and the absence of leaf necrosis provide a misleading visual indication of contamination at the site. In cases like this, the lipid biomarker would make it possible to diagnose soil Ni and Cr contamination early.

To summarize, the present study show that the lipid biomarker values measured *in situ* were repeatable from year to year, and confirm that this biomarker would provide an early indication of a plant's exposure to metal *in situ*, and could be therefore used in addition to other well-known biomarkers to facilitate or strengthen the diagnosis of soil contamination. In addition, it was of interest to demonstrate that *L. serriola* can be used as plant model in such studies because this species is widely distributed in Europe, both in wild and urban habitats (Lebeda *et al.*, 2001 and Lebeda *et al.*, 2007).

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## Figures

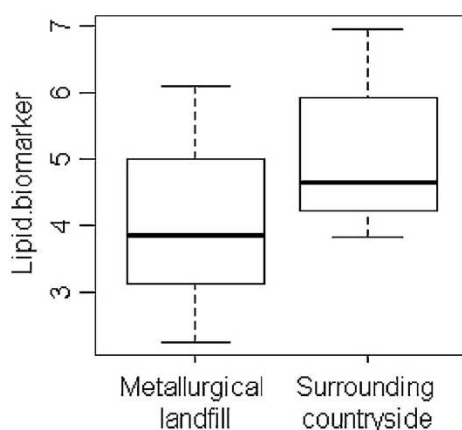


Figure 1: Distribution of lipid biomarker ( $C18:3/(C18:0 + C18:1 + C18:2)$ ) values of lettuce leaves harvested on the surrounding countryside and on the metallurgical landfill. 40 lettuce leaves were harvested on the metallurgical site (20 analyses) and on the surrounding countryside areas (20 analyses). The fatty acid composition of lettuce leaves was determined as described in Material and Methods section. The Tukey box plot show the distribution of the values of the ( $C18:3/(C18:0 + C18:1 + C18:2)$ ) ratios.  $C18:0$  = stearic acid;  $C18:1$  = oleic acid;  $C18:2$  = linoleic acid;  $C18:3$  = linolenic acid.

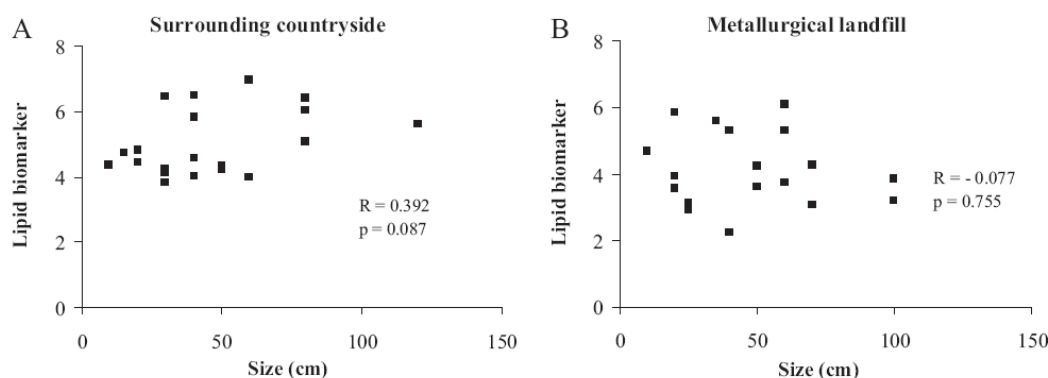


Figure 2: Correlation between the lipid biomarker and the lettuce size. Size and lipid biomarker values of lettuces harvested on the surrounding countryside (A) and on the metallurgical site (B).  $R$ : Pearson's correlation coefficient.  $p$ : significance of the Pearson's correlation coefficient.

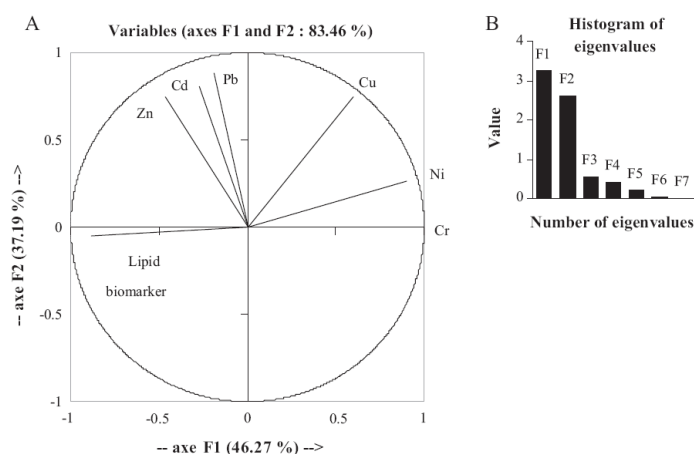


Figure 3: Correlation circle and associated vectors of 7 variables (lipid biomarker, Cu, Zn, Ni, Cr, Pb, Cd) calculated in the two first principal components of the PCA (Principal Component Analysis). (A) Correlation circle and associated vectors of variables calculated in the two first principal components of the Principal Component Analysis (PCA; the first axis [F1] explained 46.27% and the second axis [F2] 37.19% of the variance). (B) The eigenvalues of sample-based PCA showing variances of the first 7 principal components.

## Tables

Table 1: Metal concentrations measured from nine soil samples of the control area.

<i>Metals (mg kg<sup>-1</sup>) in total<sup>a</sup> fraction</i>						
	<i>Cd</i>	<i>Cr</i>	<i>Cu</i>	<i>Ni</i>	<i>Pb</i>	<i>Zn</i>
	<i>n.d.</i>	70	39	50	40	178
	<i>n.d.</i>	115	69	66	101	203
	<i>n.d.</i>	82	44	49	60	148
	<i>n.d.</i>	73	48	48	51	179
	<i>n.d.</i>	75	47	48	56	185
	<i>n.d.</i>	163	60	101	297	177
	<i>n.d.</i>	74	46	46	59	157
	<i>n.d.</i>	92	48	59	57	140
	<i>n.d.</i>	105	46	58	63	153
<i>Median</i>	<i>n.d.</i>	82	47	50	59	177

<sup>a</sup>: Extracted with aqua regia. *n.d.*: not detected

Table 2: Soil concentrations of metals and corresponding lipid biomarker values measured on nine areas of the metallurgical landfill.

<i>Location</i>	<i>Metals (mg kg<sup>-1</sup>) in total<sup>a</sup> fraction</i>						<i>Lipid biomarker</i>
	<i>Cd</i>	<i>Cr</i>	<i>Cu</i>	<i>Ni</i>	<i>Pb</i>	<i>Zn</i>	
<i>L1</i>	39.11	1130.74	4707.01	572.69	2071.53	3530.04	4.52
<i>L2</i>	18.88	960.64	443.94	518.62	778.72	4470.05	4.92
<i>L3</i>	11.47	3893.95	1080.04	2078.25	186.41	779.78	3.05
<i>L4</i>	8.19	998.47	157.38	336.47	100.06	373.87	5.61
<i>L5</i>	5.84	4085.41	983.38	2667.99	659.27	692.43	4.28
<i>L6</i>	8.56	1562.17	564.02	1523.62	868.38	1402.92	4.66
<i>L7</i>	4.52	779.62	285.63	222.33	237.88	1573.04	4.77
<i>L8</i>	2.72	3187.46	372.21	591.70	143.40	756.62	3.78
<i>L9</i>	2.80	3164.03	587.43	1312.31	229.05	470.44	4.16
<i>Median</i>	7.33	1346.46	503.98	582.20	336.66	1091.35	4.52

<sup>a</sup>: Extracted with aqua regia.

Table 3: Spearman correlation coefficient matrix between the lipid biomarker variable and the metal concentration variables (Cd, Cr, Cu, Ni, Pb and Zn).

	Lipid biomarker	Cd	Cr	Cu	Ni	Pb	Zn
Lipid biomarker	1	0.283	<b>-0.817</b>	-0.583	<b>-0.700</b>	0.183	0.200
Cd		1	-0.233	0.467	-0.017	0.583	0.600
Cr			1	0.550	<b>0.917</b>	-0.183	-0.467
Cu				1	<b>0.667</b>	0.517	0.217
Ni					1	0.117	-0.267
Pb						1	<b>0.717</b>
Zn							1

*In bold, significant values (except diagonal) at alpha = 0.050 (two-tailed test).*