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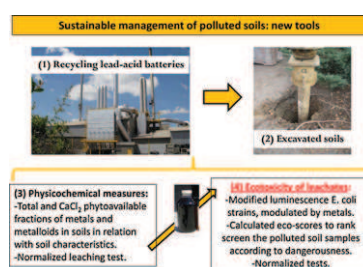[d] *Université de Nantes, UMR 6144 CNRS GEPEA, Département Génie Biologique, 18 Boulevard G. Defferre, 85000 La Roche sur Yon, France*
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GRAPHICAL ABSTRACT

ABSTRACT

Keywords:
Sustainable management of polluted soils
Metal trace elements
Ecotoxicity
Landfilling
Leaching

With the rise of sustainable development, rehabilitation of brownfield sites located in urban areas has become a major concern. Management of contaminated soils in relation with environmental and sanitary risk concerns is therefore a strong aim needing the development of both useful tools for risk assessment and sustainable remediation techniques. For soils polluted by metals and metalloids (MTE), the criteria for landfilling are currently not based on ecotoxicological tests but on total MTE concentrations and leaching tests. In this study, the ecotoxicity of leachates from MTE polluted soils sampled from an industrial site recycling lead-acid batteries were evaluated by using both modified *Escherichia coli* strains with luminescence modulated by metals and normalized *Daphnia magna* and *Alivibrio fischeri* bioassays. The results were clearly related to the type of microorganisms (crustacean, different strains of bacteria) whose sensitivity varied. Ecotoxicity was also different according to sample location on the site, total concentrations and physico-chemical properties of each soil. For comparison, standard leaching tests were also performed. Potentially phytoavailable fraction of MTE in soils and physico-chemical measures were finally performed in order to highlight the mechanisms. The results demonstrated that the use of a panel of microorganisms is suitable for hazard classification of polluted soils. In addition, calculated eco-scores permit to rank the polluted soils according to their potentially of dangerousness. Influence of soil and MTE characteristics on MTE mobility and ecotoxicity was also highlighted.

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1. Introduction

Originally located on the outskirts of cities, numerous industrial sites, sometimes abandoned, are now in urban areas and are therefore likely to have environmental and health risks to surrounding populations [1,2]. Currently, rehabilitation of the sites frequently entails excavation of polluted soils [3]. Excavated soils can thus follow two different ways: landfilling, expensive and energy intensive, or reuse/recycling, integrated to sustainable development. The choice of a specific track mainly depends on total and leachable concentrations of the pollutant in the soil [2]. Among the numerous pollutants observed in urban and peri-urban areas, trace metals are often present in soils [4]; atmosphere emissions by smelters being one of the main anthropogenic source [5,6]. MTE speciation and compartmentalization in soils can modify their impact on living organisms [5]. Now, numerous publications concluded that these two parameters are strongly influenced by soil organic matter (OM) content, pH and texture [7–9]. According to Matejczyk et al. [10], chemical weathering of soil minerals favours MTE solubilization and leachates production. Then, these leachates can pollute surrounding soils and waters. According to the council directive n°1999/31/CE, leaching tests with chemical analysis are therefore currently used for the assessment of environmental hazards of polluted soils. But, landfilling is often inevitable for strongly polluted soils, with high "hazard level" (assessed by leached and total MTE concentrations). Moreover, according to Foucault et al. [1], professionals consider the threshold set as too restrictive and they regret that excavated soils are almost always managed as waste.

In addition to the measure of total and leached MTE concentrations, it appears therefore that knowledge of MTE availability [11] and ecotoxicity may carry useful information [12–14] to improve environmental risk assessment [10]. Actually, the accurate estimation of metal phytoavailability in polluted soils and solid wastes, using single chemical extraction [15] carry interesting data to perform pertinent risk assessment and remediation efforts [16,17]. Soil quality integrates both physicochemical and biological characteristics [18]. Moreover, according to Plaza et al. [14] microorganisms play important roles in numerous soil functions. Soils are often polluted with a large variety of compounds leading to possible interactions [19], thus as reviewed by Kim and Owens [20] study of leachates ecotoxicity provides a direct functional characterization of various pollutant mixtures. But, only few studies concern the use of ecotoxicological tests to monitor contamination and bioremediation efficiency of polluted soils [21] and new tests are required by industrial sites managers to assess environmental risks. Among them, microbial bioassays offer quick, cheap and easy ecotoxicity (toxicity and mutagenicity) and bioavailability measurements on bacteria [22,23]. However, in many cases, microbial bioassays cannot be directly used for the identification and quantification of compounds due to the lack of specificity of the engineered microorganisms [24] and further studies are needed to improve these biotests.

The aim of this study was therefore to assess the ecotoxicity of leachates for landfilling of MTE contaminated soils by various complementary biotests, in addition to usual physicochemical measures. More precisely, the following two scientific objectives were aimed: (1) what is the pertinence of ecotoxicity tests to assess a more realistic human exposition to contaminated soil leachates? (2) What is the influence of soil physicochemical parameters on MTE mobility and leachates ecotoxicity? Several studies use specific bacteria strain to sense the presence of metals in soils [25–28], nevertheless, the development of statistical model to understand the link between chemical concentration of compound and bacteria sensors is still on-going work [23]. So, the originality of this study was to combine the use of new bacterial strains never tested in a context of the remediation of an industrial polluted site

and calculation of eco-scores which facilitates the comparisons between different soils.

2. Materials and methods

2.1. Soil sampling and preparation

According to Wong et al. [29], the most relevant soil layer to study the environmental and sanitary impacts of MTE in urban areas is between 0 and 25 cm. Ten top soil samples (Fig. 1) were therefore collected in the courtyard of the Chemical Metal Treatments Society (STCM), a secondary lead smelter which currently recycles batteries located in the urban area of Toulouse (43°38′12″N, 01°25′34″E). This plant was chosen because of its activity and urban location, and many data are already available [1,4–6,30]. These data allowed defining different areas in terms of environmental and sanitary risks that can vary according to past and present activities. Moreover, previous studies of the particles released in the atmosphere by Uzu et al. [5] and Schreck et al. [4] revealed the presence of several MTE (Pb, As, Cu, Cd, Zn and Sb) and gave information on the main lead speciation: PbS, $PbSO_4$, PbO—$PbSO_4$, α-PbO and Pb (by order of abundance). All soil sampling points are presented in Fig. 1; they were dried, sieved under 2 mm and treated in triplicate.

2.2. Physico-chemical analysis

pH, organic matter and limestone contents, cation exchange capacity (CEC Metson) and texture, were determined for all soil samples, respectively, according to the norms ISO 10390 [31], ISO 10694 [32], ISO 10693 [33], NF X31-310 [34] and ISO 11277 [35]. Pb, As, Cu, Cd, Zn and Sb total concentrations were measured by ICP-OES (IRIS Intrepid II XXDL) after mineralization in *aqua regia* according to ISO 11466 [36] (HNO_3 65%, HCl 37%, ratio 1:3 (v/v)). The detection limits of Pb, Cd, Sb, As, Cu and Zn were 0.3, 0.2, 0.2, 0.2, 1.3 and 2.2 $\mu g\,L^{-1}$, respectively, whereas the limits of quantification were about 0.4, 0.3, 0.4, 0.3, 2 and 3 $\mu g\,L^{-1}$, respectively. The accuracy of measurements was checked using a certified reference material 141R (BCR, Brussels). The concentrations found were within 95–102% of the certified values for all measured elements.

2.3. Leaching tests

Normalized leaching test [37] was applied to all soil samples. This procedure consisted of a single extraction with deionised water, using a solid-to-liquid ratio of 1/10. 10 g of soil (granulometry at least < 4 mm according to the norm) was mixed with 100 mL deionised water during 24 h with end-over-end agitation at 5 rpm. After centrifugation at 3000 × g during 15 min, the leachates were filtered with cellulose 0.45 μm (Millipore®) filters. 10 mL of each leachates were then acidified with HNO_3 65% prior to analysis by ICP-OES (IRIS Intrepid II XXDL, analytical errors < 5%). The other part of leachates was not acidified so as not to disturb microorganisms used for further ecotoxicological tests.

2.4. MTE phytoavailability estimate

Potentially phytoavailable MTE concentrations were estimated by $CaCl_2$ extractions according to Uzu et al. [5]. In 25 mL polypropylene centrifugation tubes, 10 mL of 10^{-2} M $CaCl_2$ were added to 1.0 g of soil. The liquid to solid ratio of 10 is high enough to avoid samples heterogeneities [38]. After agitation end-over-end during 2 h at 5 rpm at 20 °C, samples were then centrifuged during 30 min at 10,000 × g. Supernatant was sieved through a 0.22 μm mesh and acidified at 2% with HNO_3 (15 N, suprapur 99.9%). MTE concentrations were finally measured by ICP-OES (IRIS Intrepid II XXDL,
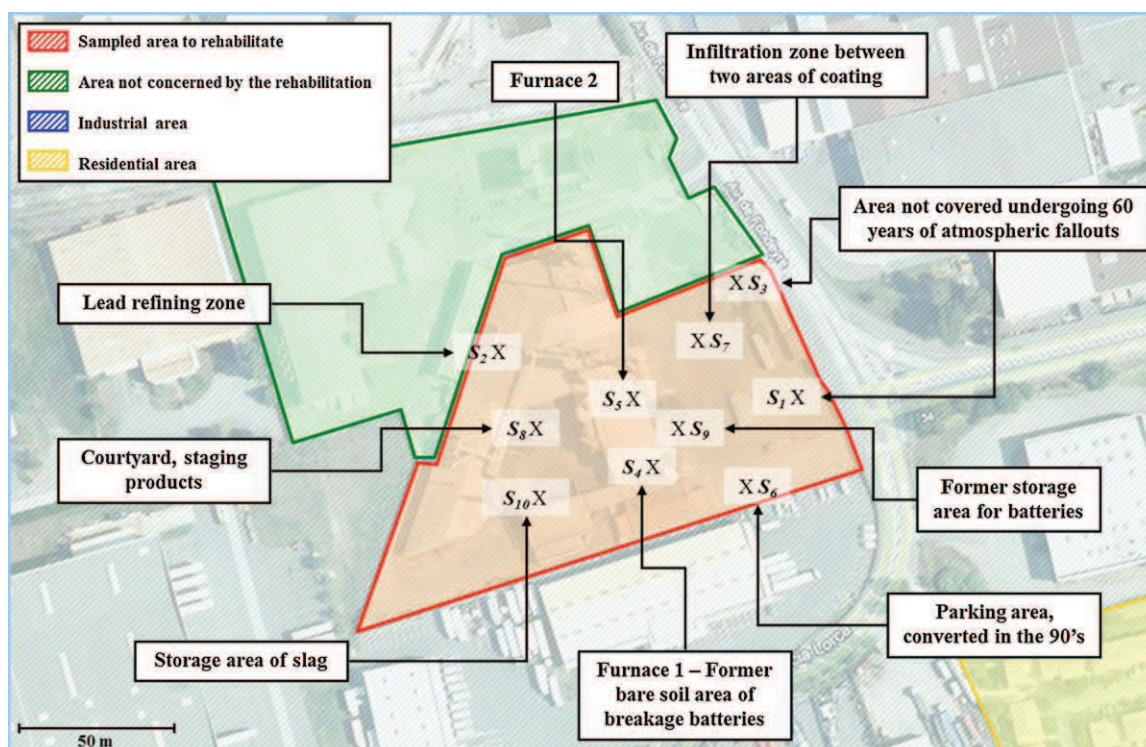
Fig. 1. Situation of the industrial study site, location and characteristics of the 10 sampling points.

analytical errors < 5%). A house reference soil was used to quality control of $CaCl_2$ extraction: this soil described by Schreck et al. [4] has the advantage of presenting the same type of contamination that the soils studied in this work. Actually, it is a soil historically polluted by battery recycling emissions ([Pb] = 1650 ± 20 mg;kg^{-1}). Using that reference soil, the detection limits of Pb, Cd, Sb, As, Cu and Zn were 0.3, 0.2, 0.2, 0.2, 1.3 and 2.2 μg L^{-1}, respectively, whereas the limits of quantification were about 0.4, 0.3, 0.4, 0.3, 2 and 3 μg L^{-1}, respectively. The concentrations found were within 95–102% of the reference values for all measured elements.

2.5. Ecotoxicity assessment

2.5.1. Daphnia magna

A first acute toxicity of leachates was performed on the water flea *D. magna* (Origin, less than 24 h old) according to [39]. Four replicates were tested for each soil solution and five neonates were used in each replicate, with 10 mL of test solution. Organisms were fed 2 h before but not during the experiment. A parafilm strip was then put on the multiwall plate placed in the incubator at 20 °C in darkness. The mobility of *D. magna* was recorded after 24 h and 48 h, and inhibition rate was calculated.

2.5.2. Microtox®

First used to assess acute ecotoxicity of metals in aquatic media [40] and normalized since 2007 [41], solid-phase Microtox® test is now currently used to evaluate the toxicity of contaminated soils or sediments [4,42,43]. The Microtox test measures the decrease in light emitted by the bioluminescent bacteria *Vibrio fischeri* after 5, 15 and 30 min of exposure [43]. In view of evaluating toxicity of collected soils, the Microtox 81.9% Basic Test with the instrument MICROTOX M 500 purchased from R-Biopharm (France) was used. 100 μL of revitalized bacteria (Lot 10J1010A) were added to each Microtox® tube, gently mixed with a pipette. 900 μL of each leachate was transferred into the glass cuvette in the Microtox® analyzer and allowed to equilibrate for 5 min before reading [44].

Light emission was recorded and the output data analyzed using Microtox® Omni software Version 1.18 [45]. All samples were tested in triplicate. Bacteria validity and the set-up of the measurement procedures were verified by reference toxin ($ZnSO_4$, $7H_2O$) according to the ISO 11348 regulation specifications [41].

2.5.3. Induction of bioluminescent bacteria

Many bacterial sensors are dedicated to the specific detection of pollutants or pollutant family. These used bio-elements are bacterial strains (*Escherichia coli*) genetically modified. In all cases, reporter genes *lux CDABE* are cloned downstream of a promoter allowing to highlight the specific or semi-specific presence for certain compounds in a sample [46,47]. In the case of metal detection, the promoters used are mostly involved in the mechanisms of bacterial resistance to heavy metals [48–50]. A set of five bioluminescent bacteria namely *E. coli* Taclux, *E. coli* Zntlux, *E. coli* Arslux, *E. coli* Coplux and *E. coli* Merlux was used in this study (Table 1). Bacterial growth and lyophilization were realized according to Jouanneau et al. [24]. At the beginning of the bioassay, the lyophilized bacteria were reconstituted with 100 μL per well of distilled water for 30 min at +30 °C. 25 μL of leachate was added to each well, and afterward the microplate was incubated for 60 min at +30 °C. Monitoring of bioluminescence was recorded using a microplate luminometer (Microlumat Plus Lb96V). The results were expressed by the logarithm of the induction ratio or the inhibition rate for the inducible strains and the constitutive strain, respectively. The induction ratio (IR) was calculated as follows:

- IR = (RLU·s^{-1})$_{i\text{-IR}}$/(RLU·s^{-1})$_{0\text{-IR}}$. (RLU·s^{-1})$_{i\text{-IR}}$ is the bioluminescence after induction with a sample, and (RLU·s^{-1})$_{0\text{-IR}}$ is the background luminescence. The inhibition rate (InR) was calculated as follows:
- InR = 1 − (RLU·s^{-1})$_{i\text{-InR}}$/(RLU·s^{-1})$_{0\text{-InR}}$. (RLU·s^{-1})$_{i\text{-InR}}$ is the bioluminescence after exposure with a sample, and (RLU·s^{-1})$_{0\text{-InR}}$ is the background luminescence.

Table 1
Observed MTE and detection limits for the different E. coli strains [24].

MTE	Detection limit of bacterial strains (standard deviation) (μM)			
	Zntlux	Arslux	Merlux	Coplux
Cd	0.0045 (0.0003)	5.9 (2.3)	0.011 (0.002)	–
Hg	0.01 (0.005)	–	1.2×10^{-7} (1×10^{-7})	–
As$^{(III)}$	28.52 (7.1)	0.256 (0.0014)	15.6 (4.3)	–
Cu	16.92 (2.9)	–	–	90.5 (11.7)
Pb	2.2 (0.6)	4.16 (0.8)	–	–
Sn	12.95 (4.24)	–	–	–
As$^{(V)}$	9.32 (1.24)	0.3 (0.06)	12.65 (5.4)	
Zn	1.7 (0.62)	–	2.3 (0.14)	–
Ni	4.4 (1.6)	–	–	–
Co	0.22 (0.014)	–	–	–
Cr $^{(VI)}$	597.2 (121.3)	–	–	–
Ag	–	–	–	2.75 (0.11)
Fe	4.34 (0.48)	–	16.1 (7.6)	–
Mn	–	–	–	–

Decision trees were designed from the learning set of bacterial bioluminescence data using the software "Metalsoft". They are specific to only one compound and organized in several binary branches. Each branch splits data according to the values of only one variable: induction ration or inhibition rate obtained from one strain. The process continues until the target value is obtained [24].

2.6. Statistical analysis

All tests were performed in triplicate and results are presented as mean $\pm$ SD (standard deviation). The statistical significance of values was checked using an analysis of variance (ANOVA) using the Statistica 9.0 package software. Each MTE-extracted concentration (both by water and CaCl$_2$ procedures) was compared to respective total concentration. Significant differences (p-value < 0.05) were measured by the LSD Fisher test.

3. Results

3.1. Physico-chemical characteristics

Soil properties are reported in Table 2. These physicochemical characteristics significantly differ in function of sample origin: it means localization on the industrial site in relation with process. pH value varied between 6.9 and 9.2, CEC value varies between 2.6 and 10.5 cmol(+) kg^{-1} and amounts of soil organic matter and carbonates (CaCO$_3$) were highly variable, respectively, from 0.9 to 46.7 g kg^{-1} and from 0 to 15.0 g kg^{-1}. MTE concentrations in polluted soil samples (Table 3) were also very heterogeneous: maximum lead concentration is 42,400 mgPb kg^{-1} and other elements are also present at high levels (up to 2095 mgSb kg^{-1}, 288 mgAs kg^{-1}, 286 mgCu kg^{-1}, 294 mgZn kg^{-1} and 80.9 mgCd kg^{-1}). All these concentrations were clearly above the national geochemical background as shown in Table 3.

3.2. Chemical extractions

MTE (Pb, As, Cu, Cd, Zn and Sb) concentrations were measured for all the solutions obtained by performing the three extractions (aqua regia, water and CaCl$_2$) on the studied polluted soil samples (Tables 3–5; Figs. 2 and 3). Leached MTE amounts in water and corresponding ratios (in comparison with aqua regia extraction, considered as "total") were significantly depending on element nature (Table 4a and Fig. 2). The highest extracted concentrations were recorded for lead, antimony and zinc (MTE with high total concentrations), respectively, 152.5, 158 and 9 mg kg^{-1}, i.e. 8.7%, 7.3% and 7.9%. In comparison, copper (at equivalent content) was significantly less extracted than zinc. Although quantitatively

low extracted ($\leq$5.4 mgCd kg^{-1}), Cd was proportionally one of the most water-soluble element (up to 15.9% for S$_6$). Arsenic was the less extracted MTE with a maximum concentration reached of 2.2 mgAs kg^{-1} (7.5% of the total for S$_4$).

CaCl$_2$ extractions results (Table 4b and Fig. 3) showed several contrasted behaviours depending both on chemical element and soil properties. The highest lead quantities extracted by CaCl$_2$ were observed for S$_1$, S$_3$ and S$_7$ (up to 178.5 mg kg^{-1}). However, for S$_4$ sample with high total lead concentration, the extracted fraction is low (1.3 mg kg^{-1}). Conversely, antimony extracted concentration reached 306.6 mgSb kg^{-1}. Other MTE showed a low extractability (in terms of quantity and ratio) whatever the sample, except
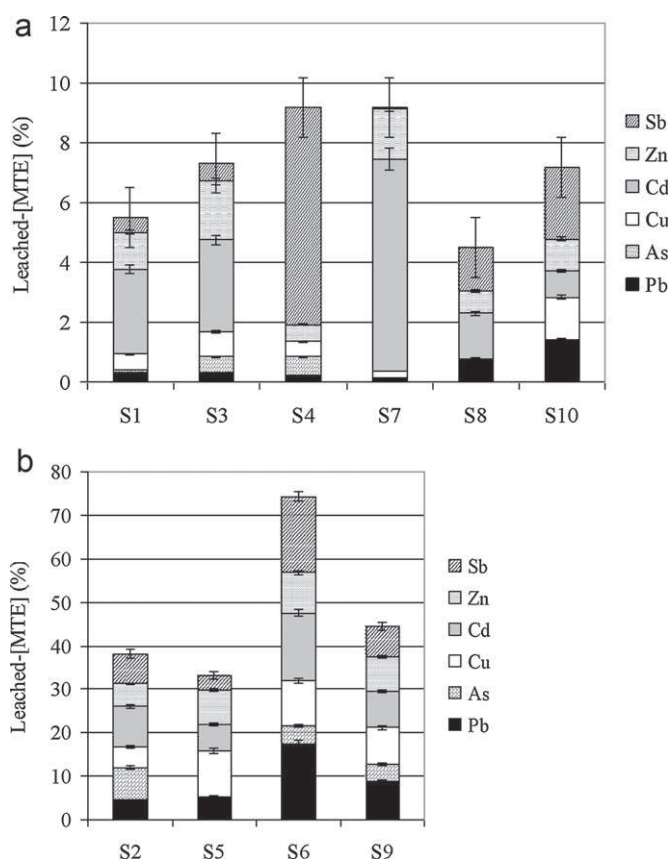

Fig. 2. Ratios (%) between MTE leached according to the EN 12457-2 procedure and aqua regia extraction, for the 10 soil samples: (a): sum of the ratios of leached-concentrations (in %) < 10% and (b) sum of the ratios of leached-concentrations (in %) > 30%.

Table 2
Main physicochemical characteristics of the ten soil samples.

Sample	pH water	Organic matter (g kg^{-1})	CEC (cmol(+) kg^{-1})	Limestone CaCO$_3$ (g kg^{-1})	Granulometry (%)				
					Clay	Thin silt	Rough silt	Thin sand	Rough sand
S$_1$	7.0	31.4	8.9	8.0	13.3	18.3	14.2	15.7	38.5
S$_2$	8.7	9.6	3.7	7.0	3.6	10.7	10.9	19.7	55.1
S$_3$	6.7	14.0	6.0	0.0	9.2	13.7	12.6	19.7	44.8
S$_4$	8.7	12.3	8.9	15.0	7.8	12.1	11.4	21.3	47.3
S$_5$	9.2	0.9	2.6	7.0	2.7	4.3	4.8	2.9	85.3
S$_6$	9.0	1.6	3.3	4.0	4.3	8.1	6.7	15.5	65.4
S$_7$	6.9	46.7	10.5	0.0	6.7	9.4	8.7	21.6	53.5
S$_8$	7.5	3.4	3.3	4.0	3.3	5.9	4.1	8.6	78.0
S$_9$	8.5	6.0	6.9	4.0	10.4	17.3	11.2	19.5	41.6
S$_{10}$	8.9	1.3	3.3	8.0	1.5	5.6	4.4	7.3	81.2

Table 3
Aqua regia MTE concentrations for the 10 soil samples (mineralization according to [29]).

MTE concentrations (mg kg^{-1})	NGB[a]	S$_1$	S$_2$	S$_3$	S$_4$	S$_5$	S$_6$	S$_7$	S$_8$	S$_9$	S$_{10}$
Pb	9–50	39,800	1425	42,400	37,250	1020	297	35,700	1445	1750	1065
As	1–25	288	28.7	51.5	52.5	5.8	9.3	34.3	8.65	13.3	10.2
Cu	2–20	286	14.7	143.5	116	13.6	16.1	249	19.4	59.5	60.5
Cd	0.05–0.45	18.4	2.24	34.3	4.15	3.39	0.69	80.9	3.39	4.7	11.3
Zn	10–100	294	37.1	216	218	42.8	41.9	545	55	116	94
Sb	0.2–10	2095	53.5	1555	2175	23.5	13.1	1955	15.9	44.5	9.15

[a] NGB: Natural Geochemical Background in France.

Table 4
Leached and phyto-available MTE concentrations (mg kg^{-1}) in the ten polluted soil samples.

Leached concentrations (mg kg^{-1})	S$_1$	S$_2$	S$_3$	S$_4$	S$_5$	S$_6$	S$_7$	S$_8$	S$_9$	S$_{10}$
(a) Leached TE concentrations (mg kg^{-1})										
Pb	127.8	63.0	126.2	85.6	53.4	51.8	51.7	11.1	152.5	14.9
As	0.29	2.17	0.28	0.33	nd[a]	0.38	nd	nd	0.54	nd
Cu	1.50	0.71	1.20	0.57	1.44	1.67	0.52	nd	5.04	0.87
Cd	0.52	0.21	1.06	nd	0.21	0.11	5.75	0.05	0.39	0.10
Zn	3.71	1.90	4.19	1.26	3.32	3.86	9.04	0.41	9.13	1.00
Sb	10.0	3.69	9.63	158.2	0.81	2.30	1.64	0.23	3.13	0.22
CaCl$_2$-extracted concentrations (mg kg^{-1})	S$_1$	S$_2$	S$_3$	S$_4$	S$_5$	S$_6$	S$_7$	S$_8$	S$_9$	S$_{10}$
(b) Phyto-available MTE concentrations assessed by the CaCl$_2$ procedure										
Pb	178.5	0.71	162.9	1.30	0.52	0.60	89.4	14.7	0.31	3.64
As	nd	nd	nd	0.17	nd	nd	nd	nd	0.04	0.01
Cu	0.43	0.20	0.37	0.38	0.19	0.18	0.32	0.21	0.29	0.64
Cd	3.16	0.20	4.16	0.22	0.31	0.22	20.1	0.68	0.22	0.24
Zn	2.77	nd	3.51	0.06	0.20	nd	25.6	0.44	0.08	0.10
Sb	1.54	0.48	1.56	306.6	0.16	0.28	0.77	0.08	0.35	0.11

[a] nd: not detected.

for S$_7$ which registered pronounced pools of Cd and Zn associated with high total concentrations. Fig. 3a and b shows the fraction of the extracted element in relation to total concentration. Cadmium appeared as the most potentially phytoavailable element. Sb and Zn also represented high extracted fractions, respectively, for S$_4$ and S$_7$. Moreover, compared to the *aqua regia* fraction, the CaCl$_2$ fraction remained lower, except for the most potentially phytoavailable Cd element (2–32%).

3.3. Ecotoxicity tests

Ecotoxicity of leachates measured by the inhibition of *D. magna* mobility was highly variable (Table 5). Whatever the sample tested, the inhibition of daphnia mobility increased between 24 h and 48 h, except for S$_3$ whose inhibition was near 100% after only 24 h. Ecotoxicity was also maximal (i.e. 100%) for S$_1$, S$_2$, S$_7$ and S$_8$ after 48 h; while the lower inhibition was observed for S$_5$ (15%). Ecotoxicity

Table 5
Results of the *Daphnia magna* and Microtox® tests for the 10 leachates.

Ecotoxicity test	Sample									
	S$_1$	S$_2$	S$_3$	S$_4$	S$_5$	S$_6$	S$_7$	S$_8$	S$_9$	S$_{10}$
D. magna[a] 24 h	90	35	100	40	10	25	80	65	45	5
48 h	100	100	100	65	15	80	100	100	65	70
Microtox®[b] 5 min	29	nt[c]	nt	nt	nt	nt	63	nt	nt	nt
15 min	78.5	nt	nt	12	nt	nt	92	nt	nt	nt
30 min	93	nt	34	34	nt	nt	96	nt	nt	nt

[a] Inhibition of mobility (%).
[b] Inhibition of bioluminescence (%).
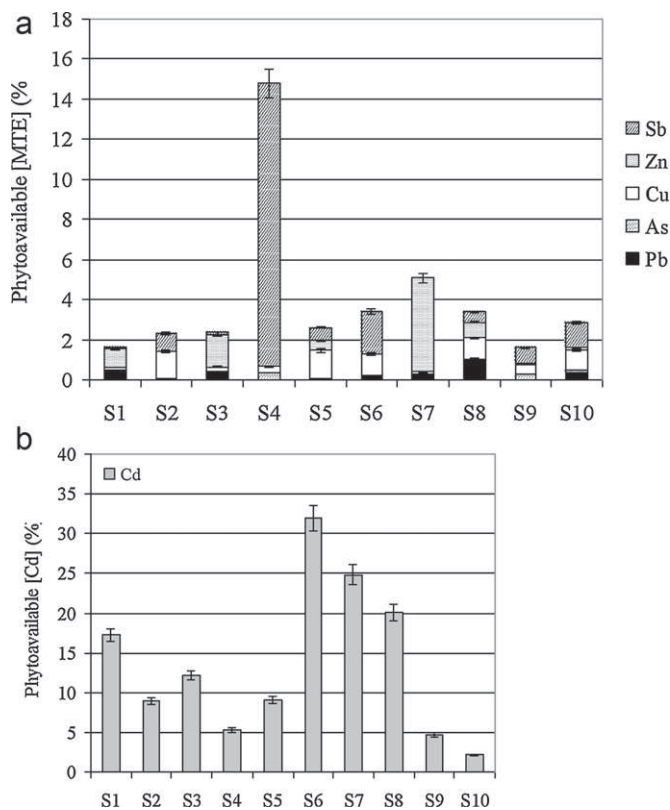[c] nt: not toxic.

Fig. 3. Ratios (%) between MTE extracted by CaCl$_2$ and *aqua regia*, for the 10 soil samples. (a) Pb, Sb, As, Zn and Cu and (b) Cd.

was not simply dependant of MTE concentration: (i) the most MTE-enriched leachates were not always the more toxicant; (ii) leachate of S$_8$ had low MTE concentrations while the inhibition of daphnia mobility was 100%.

The mean EC$_{50}$-30 min value obtained for zinc sulphate heptahydrate (expressed as Zn^{2+}) was 2.38 mg L^{-1}, allowing concluding that the invertebrates lot fulfilled the validation specifications according to [34]. Microtox® test results (Table 5) showed an increase in the number of toxic samples with the contact time: inhibition of bioluminescence was detected in two samples at the beginning of the experiment and for four of them at the end (S$_1$ and S$_7$ to 5 min; S$_1$, S$_4$ and S$_7$ to 15 min; S$_1$, S$_7$, S$_4$ and S$_3$ at 30 min). The measured ecotoxicity also increased over time and was above 90% for S$_1$ and S$_7$ after 30 min of contact. The bacteria were most affected by S$_7$ with an inhibition of the luminescence of 63% (5 min). Unlike the test on *Daphnia*, S$_2$, S$_6$, S$_8$, S$_9$ and S$_{10}$ showed no toxicity, as S$_5$ in both bioassays. As described above, S$_1$, S$_3$, S$_4$ and S$_7$ are among the most contaminated leachates ([Pb] > 80 mg kg^{-1}; [Sb] > 10 mg kg^{-1} (except S$_7$)) (Table 4a).

The sensitivity and specificity of inducible bacteria were measured after 60 min in contact with leachates. None of the sample induced the luminescence of Coplux strain. Only two samples, S$_1$ and S$_7$, showed a slight toxicity as demonstrated by the inhibition of luminescence of the constitutive strain pBtaclux (Table 6a).

These samples also induced the luminescence of Zntlux, Arslux and Merlux strains. For S$_1$ and S$_7$ the maximum IR was recorded for Arslux (IR = 99.6) and Merlux (IR = 138.1), respectively. S$_3$ increased moderately the luminescence of Merlux (IR = 12.9) and Zntlux (IR = 4.0) while S$_2$ induced only Arslux (IR = 324.9). The analysis with decision trees was then used to determine the elements potentially responsible for ecotoxicity of S$_1$, S$_2$, S$_3$ and S$_7$. Crosses between results suggested the presence of arsenic in these four leachates (up to 10^{-5} M, i.e. more than by chemical

analysis), cadmium for S$_1$, S$_3$ and S$_7$, biologically at levels lower than those measured chemically (Table 6b). Analysis of S$_7$ also showed the presence of copper and mercury (from 10^{-4} to 10^{-5} M and 10^{-9} to 10^{-5} M). According to the previous tests, these results also concluded to the ecotoxicity of S$_1$ and S$_7$, and, to a minor extent, the ecotoxicity of S$_3$ and S$_4$.

4. Discussion

4.1. Mobility and phytoavailability of MTE

In this study, soil pH were basic or close to neutral conditions and leaching procedure slightly reduced the pH by water addition. Conversely, CaCl$_2$ is already known to not modify soil pH and give results closer from field reality [15]. Thus, hazard proposed classification of polluted soils differs between water leaching and CaCl$_2$ procedures. Several studies already showed that MTE extractability is strongly influenced by the nature of the extracting agent, which can control element mobility [15,16]. Moreover, according to Dumat et al. [51] or Ferrari et al. [52], solid-liquid MTE transfers during chemical extractions are complex reactions involving numerous factors that can influence MTE speciation and release. Contact times chosen for chemical extractions were 24 h for water and only 2 h for CaCl$_2$ in accordance with the commonly used protocols: these two procedures carry complementary information but the results are not directly comparable.

All MTE were extracted in substantially equal proportions with water (from 0 to 18%); MTE concentrations in CaCl$_2$ extracts and corresponding ratios, varied in the range of those reported in the literature [5,58,61], and Cd was the most available element (up to 32%). Extracted concentrations were strongly correlated to total concentrations: R^2 = 0.92 and 0.95 ($p < 0.0001$), respectively, for water and CaCl$_2$. At the reverse side, for all the other elements no relevant correlation was found between total and extracted fractions. In agreement with previous publications [5,15], these results highlighted the influence of soil properties and MTE nature on its behaviour. Differences observed in function of MTE nature can be explained by different OM or CaCO$_3$ soil contents, CEC or soil pH. In soils, cadmium is generally easy to dissolve which explain its relatively high extractability [53]. High correlation factors were observed between exchangeable Cu and Zn fractions and soil organic matter amount: R^2 = 0.82 for Cu ($p < 0.05$) and R^2 = 0.91 for Zn ($p < 000.1$). These elements were thus less mobile because of their affinity for this soil fraction [11,54]. Concerning lead behaviour, no relationship was found between extracted and total concentrations, and the influence of even one soil parameter was difficult to highlight. Nevertheless, low extraction ratios compared to the most concentrated samples (S$_1$, S$_3$, S$_4$ and S$_7$) can be explained by stronger bounds on soil phases as mineral fraction [29]. Finally, sorption of metalloids as As and Sb, is mainly controlled by mineral phases [55]. The high Sb amount extracted from S4 could be explained not only by a higher total concentration but also by the highest CaCO$_3$ content [56]. Sb could be solubilized under the influence of soil bio-physico-chemical parameters controlling its sorption [57–59]. pH and CEC were already described as influent parameters of element extractability [60], retention and mobility in soils [11]. Thus, according to the origin of soil sampling, differences in soil parameters were observed (Fig. 1): in the industrial site, areas not covered or infiltration zones were the most impacted by MTE. Their organic matter content and CEC were also the higher, thus confirming their role in sorption/desorption mechanisms. The choice of the extractant is thereby an important step to be relevant in risk assessment and to avoid an under- or overestimation of phytotoxicity. Finally, the data obtained by chemical tests are difficult to interpret because of the many parameters interact. The realization of ecotoxicity tests to measure the impact of

Table 6
Ecotoxicity results of bioluminescence emitted by the bacterial strains.

Sample	Bacterial strain			
	ZntLux[a]	Arslux[a]	Merlux[a]	pBtaclux[b]
(a) Induction factor and inhibition rate calculated from the bioluminescence				
S_1	4.8	99.6	9.9	6.5
S_2	0.9	324.9	0.8	–
S_3	4.0	1.2	12.9	–
S_4	1.4	0.8	0.9	–
S_5	0.7	0.9	1.0	–
S_6	0.6	0.8	0.9	–
S_7	5.4	79.8	138.1	18.4
S_8	1.2	0.9	0.9	–
S_9	1.3	2.1	0.8	–
S_{10}	1.2	1.2	0.8	–

Sample	Chemical analysis				Biological analysis (prediction with decision trees)			
	Cd	As	Cu	Hg	Cd	As	Cu	Hg
(b) Comparison between MTE leached water-soluble concentrations and range "biologically" detected by the bacterial strains (unit: M)								
S_1	4.5×10^{-7}	3.7×10^{-7}	2.3×10^{-6}	–	10^{-8} to 10^{-7}	10^{-6} to 10^{-5}	–	–
S_2	1.8×10^{-7}	29.7×10^{-7}	1.1×10^{-6}	–	–	10^{-6} to 10^{-5}	–	–
S_3	9.1×10^{-7}	3.6×10^{-7}	1.8×10^{-6}	–	10^{-8} to 10^{-7}	$<10^{-6}$	–	–
S_7	48.9×10^{-7}	3.3×10^{-7}	0.8×10^{-6}	–	10^{-8} to 10^{-7}	10^{-6} to 10^{-5}	10^{-4} to 10^{-5}	10^{-9} to 10^{-5}

[a] IF: induction factor.
[b] Inhibition rate (%).

pollution on ecosystems seems therefore particularly appropriate in this type of study.

4.2. Relevance of ecotoxicity tests for risk assessment posed by landfilling

The *D. magna* ecotoxicity test was more sensitive to MTE impact than the Microtox® test. But, unlike tests on the different bacterial strains, they do not both provide information on MTE quantification. Ecotoxicity differences were measured for some samples, especially S_2 and S_8. These differences can be firstly explained by water flea sensitivity. Detection capabilities of the ecotoxicity of the leachate are actually dependent on the test used [62,63] and it has been already shown that *V. fischeri* was generally less sensitive than *D. magna* [10,64]. Instead of these tests, experiments by using bacterial strains allowed to determine and quantify the element which was potentially bioavailable and/or toxic for bacteria [50]. Then, response in ecotoxicity tests was not always directly correlated with total or water-soluble concentrations [65]. These results are in agreement with data previously obtained by Plaza et al. [14] concerning the influence of pH and CEC on MTE behaviour in soils.

Results of this study have shown that this new bioassay enables the screening of samples in terms of environmental risk during remediation process [24]. However, the drawback of the lack of specificity of one strain and the effect of a mixture of MTE (synergistic or antagonistic effects) could be overcome by using a panel of bacterial strains coupled with a predictive model [24]. Due to the lack of specific bacteria for lead, the introduction of other strains induced by lead like *Rastonia Metallidurans* AE 1433 [66] could improve the interpretation of the data.

Table 7
Hazard classification, adapted from [10,60].

Sample	Score						Class[a]	MCW[b]	W[c]	PW[d]
	D. magna	Microtox	Zntlux	Arslux	Merlux	pBtaclux				
S_1	4	3	1	3	2	2	V	4	2.5	62.5
S_2	4	0	0	4	0	0	V	4	1.3	33.3
S_3	4	1	1	1	2	0	V	4	1.5	37.5
S_4	2	1	1	0	0	0	III	2	0.7	33.3
S_5	0	0	0	0	1	0	II	1	0.2	16.7
S_6	3	0	0	0	0	0	IV	3	0.5	16.7
S_7	4	3	2	3	4	2	V	4	3.0	75.0
S_8	4	0	1	0	0	0	V	4	0.8	20.8
S_9	2	0	1	1	0	0	III	2	0.7	33.3
S_{10}	2	0	1	1	0	0	III	2	0.7	33.3

[a]

Class				MCW
I	No acute toxicity	PE < 20%	IR/InR < 1	0
II	Slight acute toxicity	20% ≤ PE < 50%	1 ≤ IR/InR < 5	1
III	Acute toxicity	50% ≤ PE < 75%	5 ≤ IR/InR < 50	2
IV	High acute toxicity	75% ≤ PE < 100%	50 ≤ IR/InR < 100	3
V	Very high acute toxicity	PE ≥ 100%	IR/InR ≥ 100	4

[b] MCW: maximum class weight score.
[c] W: class weight score.
[d] PW: class weight score in percent.

1.1. Hazard classification according to ecotoxicity

According to Persoone et al. [67] and Matejczyk et al. [10], the samples were ranked into one of five classes on the basis of the percentage effect (PE) found in Daphnia and Microtox® tests. Ranking was based on induction/inhibition rates for bacterial strains. A weight score was calculated for each hazard class to indicate the quantitative importance (weight) of the ecotoxicity in that class. The weight score was expressed as percentage.

$$\text{Class weight score} = \frac{\sum \text{all test scores}}{\text{number of tests performed} \, (= 6)}, \tag{1}$$

$$\text{Class weight score} \, (\%) = \frac{\text{class weight score}}{\text{maximum class weight score}} \times 100 \tag{2}$$

That classification system aimed at the integration of ecotoxicity data obtained in a battery of bioassays as describe by Lors et al. [68]. The classification system is based on two values: a ranking in five acute toxicity classes and a weight score for each toxicity class. The classification of the samples tested in the investigation is reported in Table 7. Samples were classified as slightly and highly toxic in 10%, toxic in 30%, and very highly toxic in 50%. The percentage of class weight class was above 5% for only S_7 and S_1 (75% and 62.5%, respectively). These samples were definitely considered as the most hazardous and acutely toxic to the microfauna. The final classification of ecotoxicity risks was $S_7 > S_1 > S_3 > S_2 = S_4 = S_9 = S_{10} > S_8 > S_5 = S_6$. Although the toxicity of some samples (S_2 and S_8, for instance) could be different depending on the test used, the ranking based on total concentrations and leachable contents of MTE was almost the same; samples S_1, S_3, S_4 and S_7 presenting the greatest risks while the less contaminated areas were generally the less hazardous. However, as our results demonstrated that only a small fraction of total MTE soil concentrations can be solubilized and phytoavailable; ecotoxicity measures complete therefore efficiently standard performed tests for a realistic risks assessment of MTE-contaminated soils accordingly to [10,14]. Moreover, the use of eco-scores improves the comparisons between bioassays and suggests the use of a restricted battery of tests to perform a cost-effective risk assessment of MTE-contaminated soils.

2. Conclusion

Biotests and eco-scores improve standard tests performed to assess risk on ecosystems induced by polluted soils. In particular, modified bacteria strains sensitive to metals are useful tools highlighting the presence of different MTE and the influence of soil parameters that lead to synergistic or antagonistic effects. Moreover, eco-scores calculation allows an easy and cheap screening of a large number of polluted soil samples and suggests a restricted battery of bioassays to perform a cost-effective risk assessment.

Nevertheless, further researches are required to develop a panel of bacterial strains specific of each MTE. Moreover, the chemical characterization of leachates could provide additional relevant insight with the use of modelling software for determination of MTE speciation and thus to evaluate the predominant parameter influencing soil availability and ecotoxicity. Finally, the procedure developed in the present work could be used in the context of bioremediation techniques like phytoremediation performed on polluted soils, to assessment their efficiency and favour their use in a context of sustainable development.

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