http://dx.doi.org/10.1016/j.chemosphere.2009.07.016

New direct contact approach to evaluate soil genotoxicity using the *Vicia faba* micronucleus test

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Keywords:
PAH
Lead
Solid medium
Ecotoxicology
Risk assessment

A B S T R A C T

A method to assess micronucleus (MN) induction in *Vicia faba* roots by direct contact exposure to a solid matrix was developed. The procedure comprised a 5-d germination period, as in the well-known method using aqueous extracts. However, the seeds were here sown directly into the test soil whereas a culture period is necessary before exposing seedlings to a liquid medium. One soil under forest and two contaminated soils from areas affected by industrial installations and a coke works were used. Three durations of direct exposure were tested: 2, 5 and 7 d. The optimal duration was evaluated at 2 d to observe maximal MN induction without observing toxicity symptoms. The methodology using aqueous extracts was applied to the same three soils: MN frequency was higher than in the direct contact assay but the ratios of MN frequencies from tested soils in comparison to the negative control were lower. However, for each soil, both the direct contact method and the aqueous extract exposure led to the same risk assessment diagnosis. The evaluation of a concentration range of a polycyclic aromatic hydrocarbons (PAH)-contaminated soil showed a dose-dependent MN frequency when the seeds were allowed to germinate before sowing in the soil: the soil genotoxicity was the highest at intermediate doses. The direct contact method was found to be rapid, sensitive and well suited to the evaluation of soil quality.

1. Introduction

Plant bioassays have been largely used to study the mutagenic effects of ionizing radiation for more than 70 years. With increasing concerns as to the genotoxicity of hazardous chemicals and pollutants in water, air and soil, several plant system bioassays have been developed for detecting genotoxicity of environmental pollutants. The tests routinely employed include the micronucleus (MN) test and chromosomal aberration assay in root tips. These techniques have been carried out in the plants *Tradescantia paludosa*, *Vicia faba* and *Allium cepa*. Among these bioassays, the *V. faba* MN test has been widely adopted for the determination of the genotoxicity of contaminated water, since a data base has been developed and bioassay protocol has been recently standardized (AFNOR, 2004).

However, regarding soil contamination, only few studies using the *V. faba* MN test have been reported (White and Claxton, 2004). Steinkellner et al. (1998) noted that by direct contact, the *V. faba* MN assay was incapable of detecting genotoxicity in metal contaminated soils readily detected by using the *Tradescantia* MN assay. Cotelle et al. (1999) noted that comparisons of MN assays in *Vicia*, *Allium* and *Tradescantia* revealed both equivalent responses in polycyclic aromatic hydrocarbons (PAH) contamination and differential responses for polychlorinated biphenyl (PCB) contamination, *Vicia* > *Allium* > *Tradescantia*. However, in this study, genotoxicity was determined by obtaining an aqueous extract of the contaminated soils. Generally, genotoxicity of contaminated soils was evaluated by adapting the water method, and by obtaining an aqueous extract of contaminated soils (Cotelle et al., 1999; Wang, 1999; Song et al., 2006). Leachates are generally prepared by shaking one unit weight of soil with ten units weight of water. This preparation strongly affects the pollutant-solid matrix relationship. Using the *Salmonella* assay, Courty et al. (2004) showed that the mutagenicity of soil extracts was influenced by different parameters such as type of solvent, temperature, duration of extraction, or soil mass/solvent volume ratio. In addition, contrasting results were found when plants were directly exposed to contact with soils or indirectly to leachates (Steinkellner et al., 1998). De Simone et al. (2000, 2005) compared the genotoxicity induced by several composts when treating *Vicia* roots with aqueous extracts, DMSO (dimethyl sulfoxide) extracts or by direct contact with soil amended with three levels of application. Results showed poor correlation between the three methods. A significant correlation only occurred between the DMSO extract and the highest level of application in the direct soil contact assessment. Recently Song

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et al. (2007) compared exposure in soil and in aqueous extracts for five PAH-contaminated soils and observed an increase of the MN frequency whatever the media, but with a lower response with the direct contact exposure.

As very few studies have compared a same contaminated soil using both direct exposure and exposure to aqueous extracts, the aim of this work was to: (i) develop a method representative of the complex nature and behaviour of soils and (ii) compare the sensitivity of the *Vicia*-MN test on contaminated soils performed using aqueous extracts or direct contact. The two methods were applied to three well-characterized soils: a non-contaminated soil under forest and two industrial soils contaminated with organic (PAHs) or metal (lead) pollutants.

2. Materials and methods

2.1. Soil samples and aqueous extract preparation

The two contaminated soil samples were collected from an industrial site (soil A) and from near a coke works (soil B) in France. Soil A was made up of a sample of taken from the immediate vicinity of a lead recycling plant (0–10 cm). Soil B was obtained by co-composting green waste and a contaminated soil collected from various depths during the site rehabilitation. Soil C was obtained from the surface layer (0–30 cm) under a forest established for over 100 years. A LUFA standard soil was used as a negative control in all direct contact experiments (Song et al., 2007). This loamy sand soil was used because for practical reasons (root tip collection) and to take into account the high organic matter content in soil B. Some key characteristics of the three soils are presented in Table 1.

Aqueous extracts were prepared by 24-h extraction with distilled water. One hundred grams of dry soil samples were mixed with 1000 mL of distilled water and stirred for 24 h at room temperature. Then, the mixture was placed at 4 °C for 24 h for decanting. For soils A and B, the pH in the leachates was in the neutral range but soil C aqueous extract presented an acidic pH (3.6), as did the soil itself (Table 1). For soil C, a second leachate was then prepared and the pH was adjusted to 6.5 with 10 M NaOH.

2.2. Chemical analyses

Extraction of metals from soil samples used an *aqua regia* digestion described in the French standard AFNOR (1995) NF ISO 11466 with a Gerhardt Kjeldatherm. Each soil sample (3 g) was digested with 21 mL of HNO_3 (65%) and 7 mL of HCl (37%). Three replicates were performed for each extraction. Heavy metals were determined by plasma optical emission spectrophotometry (ICP OES Thermo IRIS Intrepid II XDL Duo). The accuracy heavy metal assay after *aqua regia* extraction was checked by analyses of the certified material ISE 912 (soil under forest).

For aqueous extract analysis, each soil solution was filtered to 3 µm (Millipore). Heavy metals concentrations were determined by ICP OES without previous acid digestion. PAH analyses of soil B and its aqueous extract were performed by the Laboratoire Centre Atlantique, La Rochelle, France.

2.3. Vicia-MN tests

The experimental scheme developed for soil genotoxicity assessment comprised two parts (Fig. 1): testing of aqueous extracts of the different soils and secondly using different direct contact modes (time of exposure and concentration) between soils and roots. For the two exposure modes, the germination period was similar.

2.3.1. Seedling preparation

The *V. faba* seeds were prepared according to Ma et al. (1995), Cotelle et al. (1999) and El Hajjouji et al. (2007). Dry *V. faba* seeds were soaked for 24 h in deionised water, the seed coats were removed and the seeds left to germinate between two layers of moist cotton. After 5 d, the primary roots, about 2–3 cm in length were selected for the MN assay and their tips were cut off to promote the growth of the secondary roots. For each experiment, five plants were used as five independent replicates per treatment.

2.3.2. Assessment in aqueous extracts following French standard method NF T90-327

After the germination period, the primary roots of seeds were suspended in aerated Hoagland's solution. Five days were necessary to obtain secondary roots of a length suitable (1–2 cm) to be used in the test. The supernatants from decanted soil leachates were used without further treatment for exposure: secondary

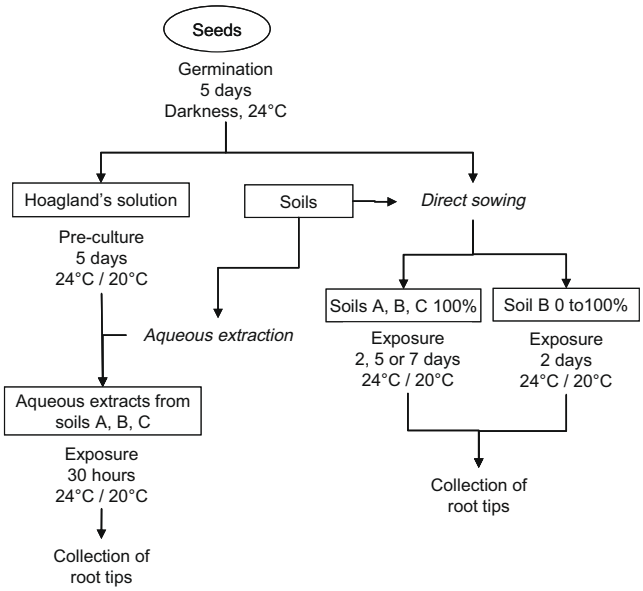

Fig. 1. Experimental sheme.

Table 1
Key properties of the soils tested.

	LUFA standard soil	Soil A	Soil B	Soil C
Organic carbon (%)	2.33	3.4	6.9	3.7
pH (soil:water, 1:2.5)	5.7	7.0	7.4	3.4
Cation exchange capacity (mval/100 g)	11	15.1	46.5	1.7
Particle size (mm) distribution according to USDA (%)				
<0.002	7.9	20.3	32.5	26.0
0.002–0.05	14.2	52.7	40.5	65.0
0.05–2.0	77.9	21.0	27.0	9.0
Soil type	Loamy sand	Calcic cambisol	Composted soil	Neoluvisol

roots were exposed to aqueous extracts for 30 h no recovery period. Maleic hydrazide (10^{-5} M in Hoagland's solution) and cadmium chloride (10^{-8} M in Hoagland's solution) were used as positive controls (PC-MH, PC-Cd). Aerated Hoagland's solution was used as negative control (NC).

2.3.3. Assessment by direct contact

The effect of the duration of seedling exposure to soil was studied by placing germinated seeds in the soil for 2, 5 or 7 d, and maintaining moisture at 2/3 of water holding capacity by introducing liquid at the bottom of the pots to avoid possible anoxia. Positive controls (PC) were made of LUFA standard soil, the moisture of which was maintained using the $CaCl_2$ or HM solutions mentioned above. Soils A, B, C and the standard LUFA soil used as negative control (NC) were wetted with distilled water.

In a second part, the direct contact method was applied to different concentrations of soil B (10%, 40%, 70 % and 100%) obtained by mixing soil B with the standard LUFA soil. Roots were collected after a 2-d exposure. Controls used LUFA soil wetted with distilled water (NC) or a 10^{-5} M solution of maleic hydrazide (PC).

2.3.4. Data collection and statistical analysis

In all experiments, at least five root tips were collected per plant that is to say at least 25 tips per treatment. Root tips were rinsed with distilled water, fixed in aceto-ethanol (1:3, v/v) at 4 °C overnight, rinsed again with deionised water for 10 min and transferred into ethanol before storage. Then, root tips were hydrolyzed with 1 N HCl for 6 min at 60 °C and squash preparations were stained with 1% aceto-orcein for 3 min at 60 °C. Five slides were prepared for each of the five seeds (one slide per root tip) and at least 1500 cells were counted per seedling, i.e. that MN frequency was obtained from at least six thousand cells per treatment. The interphase cells as defined by Ma et al. (1995) were scored for MN frequencies at 1000× magnification.

Mitotic index (MI) was expressed in % while MN frequencies were expressed per 1000 cells. In order to avoid underestimation of MN frequency due to impaired cell proliferation rate, the MN test was performed only in roots tips with a mitotic index greater than 2% (AFNOR, 2004). Statistical analysis was performed on the data: the Mann–Whitney *U*-test was used to determine the significance level against the negative control in each experimental MN test series (Béraud et al., 2007).

3. Results

3.1. Chemical analysis

Analysis results for metals and PAHs in soils and aqueous extracts are presented in Table 2. Soil A was mainly contaminated by lead (about 2000 mg kg^{-1} DM), which was weakly leached by the aqueous extract. Soil B contained only little metal and the 16 PAHs analysed were present at about 610 mg PAH kg^{-1} DM. PAHs concentrations in soil B extract were quite low with a total concentration of 595 μg L^{-1}. Heavy metals in this leachate were below detection limits except for Cu. Analyses of soil C confirmed that it was not contaminated.

3.2. Assay in aqueous extracts

The genotoxic activities of soils A, B and C evaluated with the standardized *Vicia*-MN test are shown in Table 3. In aqueous extracts, both organic and metal positive controls led to a decrease in mitotic index and a significantly increased MN frequency. On the other hand, neither soil A nor soil B reduced MI compared to the negative control while the aqueous extract from acidic soil C revealed a drastic inhibition of mitosis with an MI lower than 2% (Table 3): the MN were not counted. The aqueous extract of neutralised soil C did not show a significant decrease of the MI or an increase of MN frequency. On the contrary, both aqueous extracts from soils A and B showed a significant increase of MN frequency. The MN frequency with soil A was 2-fold higher than with soil B, and was of the same magnitude as positive controls, i.e. 10-fold higher than the negative control.

Table 2
Chemical analysis of metals and PAHs of soils (mg kg^{-1} DM) and aqueous extracts (μg L^{-1}) from samples collected on sites A, B and C.

	Soil A		Soil B		Soil C	
	Soil	Aqueous extract	Soil	Aqueous extract	Soil	Aqueous extract
As	28	n.d.	6	n.d.	5	n.d.
Cd	6	n.d.	1.2	n.d.	<l.d.	<l.d.
Cr	62	n.d.	<l.d.	<l.d.	<l.d.	<l.d.
Cu	34	157	20	75	1.1	11
Ni	33	n.d.	<l.d.	<l.d.	<l.d.	<l.d.
Pb	1933	391	35	n.d.	11.4	22
Zn	87	n.d.	80	n.d.	17.5	48
Naphtalene	n.d.	n.d.	12	1	n.d.	n.d.
Fluoranthene	n.d.	n.d.	90	47	n.d.	n.d.
Benzo(b)fluoranthene	n.d.	n.d.	84	84	n.d.	n.d.
Benzo(k)fluoranthene	n.d.	n.d.	29	27	n.d.	n.d.
Benzo(ghi)perylene	n.d.	n.d.	60	92	n.d.	n.d.
Benzo(ghi)perylene	n.d.	n.d.	24	33	n.d.	n.d.
Indeno(1,2,3-cd)pyrene	n.d.	n.d.	33	62	n.d.	n.d.
Acenaphthylene	n.d.	n.d.	4	2	n.d.	n.d.
Acenaphthene	n.d.	n.d.	8	13	n.d.	n.d.
Fluorene	n.d.	n.d.	9	2	n.d.	n.d.
Phenanthrene	n.d.	n.d.	27	8	n.d.	n.d.
Anthracene	n.d.	n.d.	59	12	n.d.	n.d.
Pyrene	n.d.	n.d.	55	50	n.d.	n.d.
Benz(a)anthracene	n.d.	n.d.	51	64	n.d.	n.d.
Chrysene	n.d.	n.d.	49	54	n.d.	n.d.
Dibenz(a,h)anthracene	n.d.	n.d.	16	44	n.d.	n.d.

n.d.: not determined; <l.d.: not detectable.

Table 3
Results of the *Vicia*-micronucleus test on soil aqueous extracts.

	Mitotic index/100 cells ± SD (n = 5)	MN/1000 cells ± SD (n = 5)
Negative control (Hoagland's solution)	9.6 ± 3.2	2.2 ± 0.9
Positive control MH (MH 10^{-5} M)	6.1 ± 2.0	22.1 ± 7.9*
Positive control $CdCl_2$ (Cd 10^{-8} M)	5.9 ± 1.5	30 ± 16.3*
Soil A	15.0 ± 3.5	18.5 ± 4.4*
Soil B	14.0 ± 4.7	9.0 ± 3.3*
Soil C (unchanged acidic pH)	0.8 ± 0.3	n.d.
Soil C (NaOH neutralized pH)	10.5 ± 1.9	2.3 ± 1.4

* Statistically significant at the 0.05 level.

Table 4
Results of the *Vicia*-micronucleus test by direct contact after 2, 5 and 7 d.

	Mitotic index/100 cells ± SD (n = 5)	MN/1000 cells ± SD (n = 5)
Direct contact – 2 d		
Negative control (loamy sand)	13.5 ± 3.1	0.08 ± 0.18
Positive control MH (MH 10^{-5} M)	7.3 ± 4.3	24.65 ± 7.50*
Positive control Cd ($CdCl_2$ 10^{-8} M)	11.7 ± 5.1	0.95 ± 0.55*
Soil A	12.5 ± 2.2	0.84 ± 0.43*
Soil B	9.0 ± 3.3	0.99 ± 0.23*
Soil C	8.5 ± 0.9	0.2 ± 0.3
Direct contact – 5 d		
Negative control (loamy sand)	20.3 ± 3.7	0.12 ± 0.29
Positive control MH (MH 10^{-5} M)	21.3 ± 4.3	9.78 ± 6. 21*
Positive control Cd ($CdCl_2$ 10^{-8} M)	18.8 ± 3.1	1.23 ± 0.96
Soil A	17.6 ± 3.2	0.81 ± 0.47
Soil B	18.5 ± 1.9	0.32 ± 0.37
Soil C	n.d.	n.d.
Direct contact – 7 d		
Negative control (loamy sand)	11.6 ± 3.1	0.13 ± 0.28
Positive control MH (MH 10^{-5} M)	10.2 ± 1.5	1.4 ± 0.7*
Positive control Cd ($CdCl_2$ 10^{-8} M)	16.1 ± 2.5	0.27 ± 0.37
Soil A	11.5 ± 2.8	0.42 ± 0.61
Soil B	13.0 ± 5.4	0.34 ± 0.39
Soil C	n.d.	n.d.

* Statistically significant at the 0.05 level.

3.3. Assay by direct contact

Whatever the exposure duration, the mitotic index was higher than 2% for all soils (Table 4). From the 5th day of exposure, soil B showed toxicity symptoms in the aerial parts with a decrease of leaf biomass (66%, data not shown).

On the 2nd day, MN frequency increased significantly for all soils, except for the soil C (Table 4). The increase was greater for organic contaminated soils (PC-MH and soil B). After a 5-d exposure, the MN frequencies decreased and were no longer significantly different from the NC except for PC-MH but the MN frequency was 2-fold lower than after 2 d, with a markedly increased standard deviation. After 7 d of direct contact, all MN frequencies decreased and only the value in PC-MH was significantly different from the NC. As no effect was observed for soil C after 2 d, MN induction was not assessed after 5- and 7-d exposures.

The results of the *V. faba*-root-MN test on four concentrations of soil B are presented in Table 5. The MI was greater than 2% for all soil concentrations. For the 10% concentration, no effects were observed on MN frequency. At higher concentrations, MN frequencies significantly increased. The most marked effect was observed in *V. faba* roots exposed to 40% soil B: MN frequency was then 25-fold higher than the negative control, but 6-fold lower than the positive control.

Table 5
Mitotic index and MN frequency values in *Vicia faba* root exposed by direct contact for 2 d to different dilutions of soil B.

	Mitotic index/100 cells ± SD (n = 5)	MN/1000 cells ± SD (n = 5)
Negative control (loamy sand)	10.5 ± 2.9	0.3 ± 0.4
Positive control (MH 10^{-5} M)	8.5 ± 4.5	26.6 ± 7.3*
10%	10.4 ± 4.1	0.7 ± 0.7
40%	14.0 ± 2.5	4.5 ± 1.8*
70%	11.2 ± 4.5	2.3 ± 1.4*
100%	10.3 ± 1.4	1.2 ± 0.4*

* Statistically significant at the 0.05 level.

4. Discussion

The genotoxicity of soils or solid matrices on *V. faba* has been largely studied using different methodologies including direct contact, contact with aqueous extracts, and DMSO extracts, with varying exposure durations. Some compared two kinds of exposure (Song et al., 2007; Chandra et al., 2004) or two kinds of pollutants, mainly organic and inorganic compounds (Cotelle et al., 1999). In view of the contrasting results obtained, an attempt has been made to compare exposures in raw solid medium and in aqueous extract for soils contaminated with organic or inorganic pollutants as well as to define a reproducible soil medium protocol.

In a first part, the duration of exposure by direct contact in raw soils was studied. Direct exposure of root tips in contaminated soils for 2 d showed a significant increase of MN frequency whatever the soil tested. In contrast, 5- and 7-d exposures did not reveal significant genotoxic effects. These results indicate the importance of the

duration of direct exposure in the MN induction. Thus, exposure time variations might explain the contrasting results reported in the literature on exposure of the roots to contaminated solid media (Minissi et al., 1998; Steinkellner et al., 1998; De Simone et al., 2000; Chandra et al., 2004; Jain et al., 2004; Song et al., 2007). According to Song et al. (2007), only results observed after 2 d of exposure in soil led to a diagnosis in a context of risk assessment similar to the standardized method with aqueous extracts. These data also underline the genotoxic potential of lead, as previously observed by Steinkellner et al. (1998).

In addition, our results demonstrated the potential genotoxicity of PAH in the two exposure conditions. These results are consistent with previous studies conducted with PAH-contaminated soils (Cotelle et al., 1999; Song et al., 2006, 2007). Cotelle et al. (1999) only observed a genotoxic effect with high dilutions of an aqueous extract, of a PAH-contaminated soil, in which the PAH concentrations were in the same range as in the present study. Moreover, these authors showed that it was difficult to find the appropriate dilution to evaluate the genotoxic potential of contaminated soils when using aqueous leachates.

Similarly, various authors have observed that MN induction varies with the soil dilution factor (Minissi et al., 1998; Jain et al., 2004; Chandra et al., 2004; De Simone et al., 2000). From these diverse results obtained by direct exposure, it appears that a dose-dependent response is obtained when the roots are exposed after a previous germination period. In contrast, no correlation was found when the seeds were allowed to germinate directly in the tested soil. In the present study, the results revealed dose-dependent MN induction (Table 4). The maximum effect was observed with the 40% soil B dilution after 2 d of exposure. These findings confirm that a dose-dependent effect can be observed when seedlings and not seeds are exposed. Moreover, the direct contact test is sensitive and easy to carry out with respect to the pollutant concentration range.

It should be noted that, following a direct contact exposure, MN frequencies were generally lower than after a hydroponic exposure: for the NC treatments, more than 2 MN appeared in Hoagland's solution per 1000 cells while less than 1 MN was observed in the LUFA soil. The maleic hydrazide-spiked soil was the only one to present a similar response for the two exposure modes (Tables 3 and 4). The low sensitivity of *V. faba* exposed by direct contact in comparison to *Tradescantia* has already been reported (Steinkellner et al., 1998). The *V. faba* beans were, in this case, previously grown in tap water for 3 d to obtain newly germinated roots. In our experimentations, as previously described by Jain et al. (2004), *V. faba* seeds were germinated on a moist cotton bed in an incubator at 24 °C until the primary roots reached 3–5 cm. The primary roots were then cut off to promote growth of lateral roots. Seedlings were then transferred to the different soils. As a consequence, the differences in terms of sensitivity and intensity of the MN frequency might be explained by the root physiology in either a solid or a liquid medium. Enstone and Peterson (2005) showed that the suberin lamella formation was earlier and more extensive in a vermiculite medium than in a hydroponic medium which depressed suberization of maize roots. Such anatomical differences might explain a variability in root permeability and the possibility for xenobiotics to enter the root cells more easily. This change would then modify the order of magnitude of the MN frequency in *V. faba* roots. Other hypotheses associated to the medium properties can also be suggested to explain the difference of MN induction between the two modes of exposure. For example, in soil, the buffer capacity and the porosity of the medium may limit the bioavailability of pollutants to the roots. Chandra et al. (2004) compared identical dilutions of a tannery leachate in water or in soil. Chromosomal aberrations and mitotic aberrations were 2-fold higher in aqueous medium than in the solid medium, indicating that soil interferes with pollutant behaviour. Finally, the assessment of the non-contaminated soil "C" with both methods showed that the direct contact test is representative and avoids skewing due to the extraction procedure especially concerning pH or non-extractable compounds. The direct contact method integrates the soil properties such as the anticlastogenic effect of certain humic substances (Ferrara et al., 2000).

In conclusion, for genotoxicity assessment by direct contact, a 2-d exposure duration, following a 5-d germination period, was shown to be the most suitable method. This test led to conclusions that were identical to those reached with the French normalized method regarding the induction of a genotoxic effect. Moreover, this mode of exposure presents advantages for risk assessment in contaminated soils: (i) it is rapid (no need for a growth period between germination and exposure); (ii) it seems sensitive and reproducible over a wide range of pollutant concentrations; and (iii) it takes into account the soil properties and the presence of non-water-extractable compounds. The direct contact exposure mode was found to be efficient to assess the genotoxic potential for *V. faba* of soils and solid matrices contaminated with organic and/or metal pollutants. However, the proposed method should now be validated by testing a higher number of soils and contamination types.

Acknowledgements

This work was supported by the French National Agency for Research and Technology (ANRT, Grant no. 789/2003), by the Association for the Environment and Security in Aquitaine (APESA) and by the French National Agency of Wastes and Energy (ADEME, Grant no. 0375C0114).

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