Biomonitoring of the genotoxic potential of aqueous extracts of soils and bottom ash resulting from municipal solid waste incineration, using the comet and micronucleus tests on amphibian (*Xenopus laevis)* larvae and bacterial assays (Mutatox® and Ames tests)

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Abstract

The management of contaminated soils and wastes is a matter of considerable human concern. The present study evaluates the genotoxic potential of aqueous extracts of two soils (leachates) and of bottom ash resulting from municipal solid waste incineration (MSWIBA percolate), using amphibian larvae (*Xenopus laevis*). Soil A was contaminated by residues of solvents and metals and Soil B by polycyclic aromatic hydrocarbons and metals. MSWIBA was predominantly contaminated by metals. Two genotoxic endpoints were analysed in circulating erythrocytes taken from larvae: clastogenic and/or aneugenic effects (micronucleus induction) after 12 days of exposure and DNA-strand-breaking potency (comet assay) after 1 and 12 days of exposure. In addition, in vitro bacterial assays (Mutatox® and Ames tests) were carried out and the results were compared with those of the amphibian test. Physicochemical analyses were also taken into account. Results obtained with the amphibians established the genotoxicity of the aqueous extracts and the comet assay revealed that they were genotoxic from the first day of exposure. The latter test could thus be considered as a genotoxicity-screening tool. Although genotoxicity persisted after 12 days' exposure, DNA damage decreased overall between days 1 and 12 in the MSWIBA percolate, in contrast to the soil leachates. Bacterial tests detected genotoxicity only for the leachate of soil A (Mutatox). The results confirm the

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ecotoxicological relevance of the amphibian model and underscore the importance of bioassays, as a complement to physico-chemical data, for risk evaluation.

Keywords: *Xenopus laevis*; Genotoxicity; Micronucleus test; Comet assay; Ames test; Mutatox; Contaminated soil; Leachate; Municipal solid waste incineration bottom ash; Percolate

1. Introduction

Environmental management of municipal solid wastes and contaminated sites is an important economic and environmental problem. The regulations relative to their management are insufficient, as regards the precise evaluation of the future evolution and possible effects on ecosystems of these contaminated matrices. There is no clearly defined consensus concerning the strategy for assessing the ecological hazard of these soils and wastes.

In the early 1990s, the French waste policy aimed at an ambitious reduction of domestic waste deposits in landfills. Recently, storage in Technical Burying Centres has been given a high priority because of the potential contamination of domestic wastes. Since 2002, in application of the law of the thirteenth of July 1992 on waste removal and material salvage, final wastes must be deposited in refuse dumps. Consequently, municipal solid wastes (MSW) are dealt with mainly in Incineration Centres. Combustion techniques reduce MSW by 80%. However, the procedure generates new solid residues, including municipal solid waste incineration bottom ash (MSWIBA). Since MSWIBA contain high concentrations of metals, they pose potential environmental problems. Moreover, their increasing use in road construction makes it urgent to study their contribution to the pollution of surrounding receptor compartments (watercourses, soils, lentic medium, etc.). At present, their industrial use is based on criteria which are defined by the French ministerial circular (M.A.T.E., 1994) relative to the removal of urban incineration residues. However, these criteria do not ensure their ecological compatibility and do not take into account their genotoxic potential.

Likewise, the management of polluted sites is also a significant economic and environmental problem. In 2003, 3662 polluted sites were localized, 66.96% of which had led to soil or ground water pollution (M.A.T.E., 2003). Soil pollution is readily mobilised (generally finishing up in the aquatic environment) and can affect exposed populations.

Consequently, the presence of pollutants in MSWIBA and soils can produce genotoxic activity in fresh water and their effects on the ecosystems and on human health is an area of increasing concern. Thus, in the present study, a laboratory tool (lysimeter) has been developed to reproduce the conditions of bottom ash utilisation and to study the environmental impact of this waste via the percolates collected at the bottom of the lysimeter. The genotoxic impact of the contaminated soils was measured on leachates by means of the French standard leaching test NF X31-210 (AFNOR, 1992).

The genotoxicity of unknown matrices is usually evaluated by placing them in contact with organisms which are then examined for genetic damage. A number of tests have been developed, using aquatic animals (see the reviews of Jaylet et al., 1990; Jaylet and Zoll, 1990; Zoll et al., 1990; Godet et al., 1993), which can potentially be used to assess the genotoxic potency of aqueous extracts. The tests can be carried out with intact animals, taking into account uptake and elimination, internal transport and metabolism. An example is the use of amphibians, which have proved to be valuable biomarkers and sensitive models for environmental studies (Ferrier et al., 1998; Djomo et al., 2000; Békaert et al., 2002; Gauthier et al., 1993; Gauthier, 1996).

We used the amphibian *Xenopus laevis* to monitor the effects of exposure to aqueous extracts from soils (leachate) and MSW (percolate). Two genotoxic endpoints were analysed in larvae of these animals: micronucleus induction and DNA-strand-breaking potency (comet test) in the circulating blood.

Micronucleus formation, which is detected with the micronucleus test (MNT), is the consequence of chromosome fragmentation and/or dysfunction of the mitotic apparatus. Thus, clastogenic compounds

and/or spindle poisons both lead to an increase in the number of micronucleated cells. Primary DNA damages, which are detected using the comet assay (CA), are double and single DNA strand breaks, alkaline-labile-sites, incomplete excision-repair-sites and crosslinks. Among aquatic vertebrates, amphibians have already proved to be useful biological models for micronucleus (Jaylet et al., 1986; Krauter et al., 1987) and primary DNA damage induction (Clements et al., 1997; Mouchet, 2002; Mouchet et al., 2005, in press) in erythrocytes.

The main objective of our study was to assess the genotoxicity of two soil leachates and MSW percolate with respect to the amphibian *X. laevis* larvae. The MNT and CA were compared with respect to their sensitivity. In addition, the results of in vitro bacterial assays (Ames and Mutatox assays) were compared and chemical results were taken into account.

2. Materials and methods

2.1. Municipal solid waste incineration bottom ash (MSWIBA), contaminated soil, sampling methods, aqueous extraction and chemical analyses

2.1.1. MSWIBA

MSWIBA was provided by a French Municipal Solid Waste Incineration Plant (MSWIP) which is located on a confidential site. The combustion was performing between 1000 and 1100 °C at a rate of 7.5 tons an hour. MSWIBA was then cooled by water immersion and stocked in the open air on a platform to mature. After a month, MSWIBA were defined as valorizable according the ministerial circular (94-IV-1) of the 9th May 1994 (classification is based on the pollutant potential of MSWIBA according to lixiviation assays; AFNOR X 31-210, 1992). MSWIBA were finally left at room temperature protected from air and light.

2.1.2. Aqueous extraction

MSWIBA was placed in lab lysimeters. An aqueous extract (MSWIBA percolate, P_{MSWIBA}) was obtained by flowing reconstituted rain-water (distilled water) under gravity through layers of MSWIBA. Five liters of water was applied daily to the surface of the lysimeter (200 kg) for 20 days, late in the afternoon in order to limit evaporation (final ration: 0.5). Free water flow was facilitated by the presence of a sand layer. Below the MSWIBA block, a geotextile (that retain the fines and prevented dispersion in leachates) was fixed on the gravel-bed (to expedite water flow). MSWIBA was deposed in layers of 3–4 cm deep. Each layer was compacted with the «Optimum Proctor Normal» conditions (optimum compaction of MSWIBA for use in road construction).

2.1.3. Contaminated soils

Two soils from confidential French areas were chosen according to their pollutant load. Both correspond to industrial waste lands. Soil A (S_A), from an excavated site, was collected at six different stations of the area of storage (1 m^2 of area and 2.5 m of depth) that was plant-free. The sample was dried at room temperature in order to reduce the humidity level from 88.5% to 10%. Soil B (S_B) came from an industrial area of cokering that was covered with plants. Three tons were filtered through 5 cm. The sample was dried at room temperature in order to reduce the humidity level from 92.3 to 10%. Both soils were manually crushed, filtered through 4 mm and finally homogenised in a concrete-mixer. S_A was contaminated by residues of solvents and metals. S_B was mainly contaminated by polycyclic aromatic hydrocarbons (PAHs) and metals.

2.1.4. Aqueous extraction

Aqueous extracts were obtained according to the static leaching procedure (AFNOR, 1992) without filtration at 0.45 μm (in order to preserve adsorbed contaminants on solid matters in suspension that are potentially available for the amphibian larvae; Békaert, 1999). Moreover, filtration through 0.45 μm pores is not representative of natural phenomena such as soil leaching. The soil (1 kg dried matter) was mixed with 10 L of ultra-pure water and stirred for 24 h. After 2 h of decantation, the leachates were collected by siphoning.

Forty liters of each aqueous extract was collected in 5-L plastic containers for subsequent laboratory tests with amphibian larvae. Leachates were maintained at 4 °C in the dark before testing and were homogenised before use. The required volumes were brought to a temperature of 22 ± 0.5 °C.

2.1.5. Chemical analyses

Organic contaminants (polycyclic aromatic hydrocarbons PAHs, polychlorinated biphenyls PCBs and organochlorinated pesticides OCPs) in aqueous extracts were analyzed by capillary gas chromatography/mass spectrometry (according to French ISO 6468 and ISO 13877 procedures) after a liquid–liquid extraction using a dichloromethane/hexane mixture (according to 3545 US-EPA procedure (US-EPA, 1996)). Metals were analyzed by atomic absorption spectroscopy after mineralization in a microwave oven under acid (HNO_3) conditions.

2.2. Amphibian test procedures

2.2.1. Xenopus, breeding and maintenance

Sexually mature *Xenopus* were provided by the Developmental Biology Department, Paul Sabatier University (Toulouse, France). Males were injected with 400 IU human chorionic gonadotropin (HCG) and females were injected with 700 IU HCG. Viable eggs were maintained in an aquarium containing mineral water at 22 ± 0.5 °C until they reached a development stage appropriate for experimentation (usually 3 weeks).

2.2.2. Exposure conditions

The conditions of exposure, basically the same for the MNT and the CA, are described in the French Standard AFNOR NF T90-325 (AFNOR, 2000). AFNOR is the French National Organization for quality regulations. Exposure began with larvae at stage 50 of the *Xenopus* table of development (Nieuwkoop and Faber, 1956). L_A, L_B and P_{mswi} were analysed using larvae from three different layings. For one MNT and CA test, the larvae were taken from the same hatch to reduce inter-animal variability. They were exposed in groups of 20 animals (100 mL/larvae) in 5-L glass flasks containing either the control medium (negative and positive control) or the test medium (aqueous extract, pure or diluted in mineral water). The negative control was mineral water. The positive control for the MNT was Benzo[a]pyrene (B[a]P, [50-32-8], Sigma France) at 0.125 mg/L, first dissolved in DMSO then added to the mineral water. For the CA, the positive control was methyl methanesulfonate (MMS, [66-27-3], Sigma France) at 1.56 mg/L (Mouchet, 2002). Acute toxicity (death, reduced size, diminished food intake, abnormal behaviour) to the treated larvae was examined by visual inspection to ensure that both genotoxicity tests were performed under conditions that were not acutely toxic. The concentrations tested for the aqueous extracts were: 0, 1.5, 3 and 6% for the leachate of soil A (L_A); 0, 6, 12 and 25% for the leachate of soil B (L_B); 0, 3, 6 and 12% for the percolate of MSWIBA (P_{MSWIBA}). The larvae were subjected to a 12-h light/12-h dark cycle and were fed every day on dehydrated aquarium fish food. The flasks were partially immersed in water baths to maintain the temperature at 22 ± 0.5 °C. The media in control and experimental flasks were renewed daily.

2.2.3. Micronucleus and comet assay procedures

At the end of the period of exposure, respectively 12 days (MNT) and 1 and 12 days (CA), a blood sample was obtained from each anaesthetized larva by cardiac puncture and used for both tests. Genotoxicity was assessed up to the highest concentration that did not lead to signs of acute intoxication of the exposed larvae.

2.2.4. MNT

The *Xenopus* MNT was performed according to the Standard AFNOR NF T90-325 procedure (AFNOR, 2000). After 12 days of exposure, a single smear of blood was prepared from each animal. After fixing in methanol and staining with hematoxylin, the smears were screened under the microscope (oil immersion lens, $\times 1500$). The number of erythrocytes that contained one micronucleus or more was determined in a total sample of 3000 erythrocytes per larva.

For each group of animals, the results (number of micronucleated erythrocytes per thousand, MNE ‰) obtained for the individual larvae were arranged in increasing order of magnitude and the medians and quartiles calculated. The statistical method used to compare the medians was based on the recommendations of McGill et al. (1978) and consists in determining the theoretical medians of samples of size n (where $n \geq 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times \mathrm{IQR} / \mathrm{v}n$, where M is the median and IQR is the Inter-Quartile Range. Under these conditions, the difference between the theoretical medians of the test groups and the theoretical median of the negative control group is significant to

within 95% certainty if there is no overlap. The result is then positive.

2.2.5. CA

The CA was performed essentially according to the procedure first described by Singh et al. (1988) and subsequently adapted to *X. laevis* larvae (Mouchet, 2002). Frosted microscope slides were precoated with freshly prepared normal melting agarose (NMA, 0.8% in phosphate buffered saline PBS), left at room temperature to allow the agarose to dry. After cardiac puncture, an aliquot of heparinized blood cell suspension was immediately diluted 50 fold in PBS. Erythrocyte viability was routinely determined using the Trypan blue exclusion test and samples showing less than 90% of viability were discarded (Collins, 2002). Diluted blood was mixed with an equal volume of fresh, low-melting-point agarose (LMA, 1% in PBS). Sixty-five microliters of this agarose-cell suspension was spread on precoated slides (2 per animal) and covered with a coverslip. After cooling for 7 min on ice, the coverslip was gently removed and a third layer, consisting of 90 μL of LMA (1% in PBS), was then added and allowed to solidify for 7 min on ice before gently removing the coverslip. The slides were then immersed in freshly prepared ice-cold lysing solution (2.5M NaCl, 0.1M Na_2EDTA, 0.01M Tris, 1% Triton X-100, 10% DMSO, adjusted to pH 10 with NaOH) and left for 1 h. Lysis and subsequent steps were performed in the dark under dim red light. The slides were then removed from the lysing solution and transferred to a horizontal electrophoresis tank containing cold freshly prepared alkaline buffer (0.3M NaOH, 1 mM Na_2EDTA, pH >13) for 20 min at 4 °C in order to allow the DNA to unwind. Electrophoresis was carried out in the same buffer for 20 min by applying an electric field of 20 V and adjusting the current to 300 mA. Finally, the slides were washed twice in a neutralization buffer (0.4M Tris–HCl, pH 7.5) for 5 min, before being dehydrated in absolute ethanol for 5 min. Slides were analysed using a confocal microscope (LSM 410 inverted laser scan microscope, Zeiss) at 40× magnification, after staining the slides (0.05 mM of ethidium bromide solution). Quantitative assessment of DNA damage in erythrocyte nuclei was performed using the Komet 4.1 image analysis software (Kinetic Imaging Ltd.), by measuring Extent Tail Moment (ETM, defined as the tail length weighted by the percentage of tail DNA) and Tail Length (TL, length of the tail, distance between the head and the last DNA fragment). Thirty randomly selected cells were analyzed on each slide. Comet images were randomly captured from the centre of the slide, at a constant depth in the gel, avoiding the edges of the slide and overlapping figures. Comets with completely fragmented DNA (hedgehog-like figures with no apparent head) that could not be measured by the image analysis system were not taken into account. Data from 2 slides per animal and 2 animals for each concentration tested were pooled for the final processing. In order to compare the genotoxic responses obtained with the MNT and the CA, to evaluate genotoxicity with the CA at each sample time and to evaluate a dose–response relationship, it is necessary to test at least 3 doses for each time of exposure. This constraint obliged us to limit to two the number of animals tested for each dose, because using more would have entailed performing a second, independent electrophoresis, with its inevitable inherent variability. ETM and TL data (120 measurements per tested concentration) were analyzed using the non parametric Kolmogorov–Smirnov test ($\alpha = 0.05$).

Finally, in order to establish the time-course of DNA damage between days 1 and 12 of exposure at a given concentration i of draining water, we introduced the following evolution coefficient:

$$\begin{aligned} EC_{CA} = [[\{&m(\text{concentration } i,\ \text{time } 12) \\ &- m(\text{concentration } i,\ \text{time } 1)\} \\ &- \{m(\text{neg. control},\ \text{time } 12) \\ &- m(\text{neg. control},\ \text{time } 1)\}] \\ &/\{m(\text{concentration } i,\ \text{time } 1) \\ &- m(\text{neg. control},\ \text{time } 1)\}] \times 100 \end{aligned} \quad (1)$$

where m(concentration i, time 12), m(concentration i, time1) and m(neg.control, time 12), m(neg.control, time 1) represent the mean values of the parameter computed for a given draining water concentration i, and the corresponding negative control respectively at the two exposure times considered. The mean values of the parameter relative to the negative control are included in the above expression in order also to take into account the evolution of the negative control. The evolution percentage (EC_{CA}) can be positive

(increased DNA damage between days 1 and 12 of exposure) or negative (reduced DNA damage).

2.3. Bacterial test procedure

2.3.1. *Salmonella typhimurium his⁻ reverse mutation test (Ames test)*

The *Salmonella typhimurium*_his⁻ reverse mutation test was conducted with the bacterial strains TA98 and TA100. The test consists in measuring the growth of bacteria in a histidine-deficient medium. The bacterial mutants, which are initially unable to synthesize histidine and cannot grow in a medium devoid of this amino acid, revert and recover this property after exposure to mutagens. We applied the classical procedure in agar medium described by Maron and Ames (1983). Positive (nitrofluorene, sodium azide and benzo[a]pyrene) and negative controls (DMSO or sterilized water) were included in each experiment. Revertant colonies on agar plates were counted after a 48 h incubation period at 37 °C. Results were considered positive when the average number of colonies per plate was more than twice the average number found in negative controls.

In order to eliminate bacterial contamination, the aqueous samples were sterilized by membrane filtration (0.45 μm), regardless of the protocol used. The Ames test was applied with or without metabolic activation of pollutants. Microsomal enzymes were prepared from rats (Sprague–Dawley) induced with Arochlor 1254, as described by Ames et al. (1975). The supernatant fraction of the liver was homogenized and centrifuged at $9000 \times g$. The supernatant of hepatic homogenate (S9) contained metabolizing enzymes and was used in bioassays for the detection of pro-mutagens.

2.3.2. *Mutatox test*

The Mutatox test, which was developed by the Microbics Company (now Azur Environmental), uses dark mutants of the luminescent bacterium *Vibrio fischeri* (stain M 169; Ulitzur et al., 1980). The assay was carried out with lyophilized reagents kept at −20 °C and reconstituted at the time of use, according to the procedure described by Microbics (1993). In the presence of mutagenic compounds, mutants can revert and recover their luminescence. Luminescence was measured with a bioluminometer after different times of exposure (16, 20 and 24 h) of the bacteria to the test medium at 27 °C. The concentration which induced a luminescence intensity at least twice that of the negative control was considered positive. The sample was deemed mutagenic when at least two successive concentrations were positive. The assays were performed in the presence and absence of S9 mix. Positive controls (9-aminoacridine and benzo[a]pyrene) were included in each series of assays. The S9 mix used in the Mutatox test was provided by Azur Environmental.

3. Results

3.1. Amphibian genotoxicity (MNT and CA)

3.1.1. Soil leachates

With respect to micronucleus formation in amphibian larvae after 12 days of exposure (Table 1), L_A proved to be genotoxic down to a concentration of

Table 1
Results of the *Xenopus* micronucleus assay: number of micronucleated erythrocytes per 1000 cells (MNE‰) in larvae exposed for 12 days to aqueous extracts

	Concentrations (%)						
	Nc	Pc	1.5	3	6	12	25
L_A	1 ± 0.41	14 ± 1.22	1 ± 0.20	2 ± 0.41*	4 ± 0.81*	–[b]	–[b]
L_B	1 ± 0.41	12 ± 1.82	[a]	[a]	3 ± 0.61*	4 ± 1.42*	4 ± 0.61*
P_{MSWI}	0 ± 0.41	14 ± 1.42	[a]	1 ± 0.41*	2 ± 0.81*	3 ± 0.41*	–[b]

Genotoxicity is expressed as the median values of MNE‰ and their 95% confidence limits. Asterisk indicates a genotoxic result.
L_A: leachate of soil A. L_B: leachate of soil B. P_{MSWI}: percolate of municipal solid waste incineration bottom ash. Nc: negative control (mineral water); Pc: positive control (0.125 mg/L B[a]P); [a] concentration not tested; [b] micronucleus assay was not carried out because of established acute toxicity to larvae.

Table 2a
Results of the *Xenopus* comet assay

		Concentrations (%)						
		Nc	Pc	1.5	3	6	12	25
L_A	ETM	0.16 ± 0.04	6.26 ± 1.42	1.41 ± 0.33 ↗	5.94 ± 1.01 ↗	6.77 ± 0.99 ↗	–[b]	–[b]
	TL	11.27 ± 1.59	44.60 ± 4.32	27.12 ± 2.87 ↗	52.38 ± 3.84 ↗	52.09 ± 3.53 ↗		
L_B	ETM	0.18 ± 0.04	7.55 ± 1.85	[a]	[a]	0.96 ± 0.24 ↗	2.76 ± 0.59 ↗	6.29 ± 1.25 ↗
	TL	18.68 ± 1.28	50.17 ± 5.7			22.98 ± 2.05 ↗	32.74 ± 3.4 ↗	50.28 ± 4.00 ↗
P_{MSWI}	ETM	0.09 ± 0.03	6.87 ± 2.38	[a]	0.27 ± 0.07 ↗	0.13 ± 0.03 ↗	0.13 ± 0.03 ↗	–[b]
	TL	10.32 ± 1.6	47.73 ± 7.67		13.04 ± 1.2 ↗	12.11 ± 1.21	10.92 ± 1.1	

Mean values of ETM/TL parameters in larvae reared for 1 day in aqueous extracts.
This table presents the calculated mean values of ETM (Extent Tail Moment) and TL (Tail Length) followed by their 95% confidence limits. The notation "↗" indicates an increase of DNA damage relative to the negative control, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$). L_A: leachate of soil A. L_B: leachate of soil B. P_{MSWI}: percolate of municipal solid waste incineration bottom ash. Nc: negative control. Pc: positive control (1.56 mg/L MMS). [a] Concentration not tested; [b] comet assay was not carried out because of established acute toxicity to larvae.

3%. L_B exhibited significant genotoxicity regardless of the concentration. In both cases, no obvious dose–response relationship could be perceived.

With regard to DNA damage induced after 1 day of exposure (Table 2a), leachates of both soils were genotoxic at all the concentrations tested and irrespective of the parameter measured. A dose–response relationship was observed with both parameters for L_A and L_B. After 12 days of exposure (Table 2b), and L_B remained genotoxic to the larvae, depending on the parameter measured and the concentration. L_A exhibited genotoxicity at 6% and 3%, depending on the parameter measured. L_B exhibited genotoxicity (with respect to both parameters) only at 25%. A dose–response relationship was observed for L_A with parameter TL, but no such relationship was observed for L_B. Between days 1 and 12, reduction of DNA damage was significant in each soil leachate, regardless of the concentration and the parameter measured (Fig. 1). The global response of the larvae exposed to the leachate was shown to be inversely proportional to time for both parameters.

3.1.2. MSWIBA percolate

Evaluated by micronucleus formation after 12 days of exposure (Table 1), all concentrations of P_{MSWIBA} were significantly genotoxic to amphibian larvae. A dose–response relationship was clearly apparent. With respect to induction of DNA damage, 1 day's exposure to P_{MSWIBA} (Table 2a), resulted in a level of damage (using the ETM parameter) significantly higher than the corresponding negative control, regardless of the concentration, whereas it was genotoxic only at 3% using the TL parameter. The response of the larvae to 3, 6 and 12 mg/L of P_{MSWIBA} was found to be inversely dose-dependent. After 12

Table 2b
Results of the *Xenopus* comet assay

		Concentrations (%)						
		Nc	Pc	1.5	3	6	12	25
L_A	ETM	0.13 ± 0.03	16.17 ± 3.91	0.15 ± 0.03	0.21 ± 0.16	0.33 ± 0.06 ↗	–[b]	–[b]
	TL	11.03 ± 1.32	55.10 ± 5.5	10.26 ± 1.02	13.8 ± 1.2 ↗	17.45 ± 1.43 ↗	–[b]	–[b]
L_B	ETM	0.13 ± 0.06	4.98 ± 1.38	[a]	[a]	0.17 ± 0.06	0.16 ± 0.05	0.56 ± 0.13 ↗
	TL	12.13 ± 1.54	40.18 ± 4.71	[a]	[a]	12.72 ± 1.31	12.06 ± 1.2	17.98 ± 1.32 ↗
P_{MSWI}	ETM	0.07 ± 0.01	3.93 ± 0.9	[a]	0.49 ± 0.11 ↗	0.39 ± 0.14 ↗	0.27 ± 0.07 ↗	–[b]
	TL	7.27 ± 0.9	37.63 ± 4.25	[a]	15.17 ± 1.39 ↗	16.49 ± 2.17 ↗	14.24 ± 1.66 ↗	

Mean values of ETM/TL parameters in larvae reared for 12 days in aqueous extracts.
This table presents the calculated mean values of ETM (Extent Tail Moment) and TL (Tail Length) followed by their 95% confidence limits. The notation "↗" indicates an increase of DNA damage relative to the control negative, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$). L_A: leachate of soil A. L_B: leachate of soil B. P_{MSWI}: percolate of municipal solid waste incineration of bottom ash. Nc: negative control. Pc: positive control (1.56 mg/L MMS). [a] Concentration not tested; [b] comet assay was not carried out because of established acute toxicity to larvae.

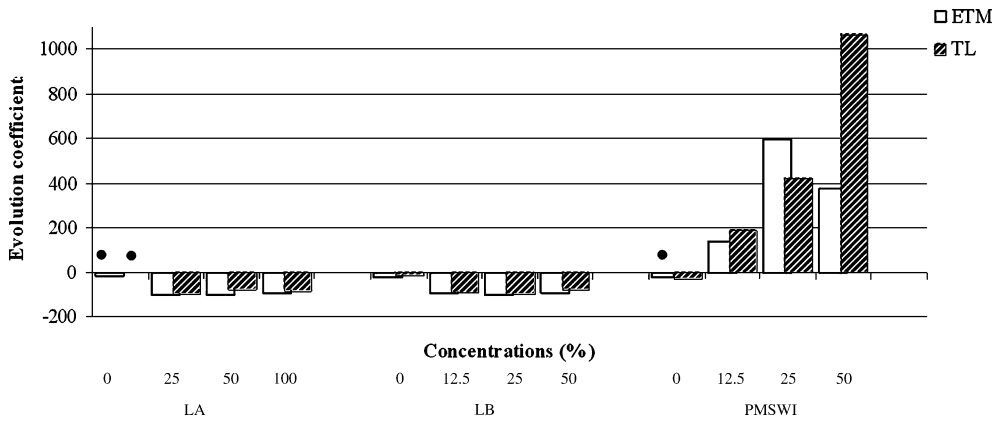

Fig. 1. Results of the *Xenopus* comet assay. Evolution coefficients (EC_{CA}) of ETM and TL in larvae reared in aqueous extracts between days 1 and 12 of exposure. The evolution coefficients were calculated as mentioned in Section 2.2 (see Eq. (1)) Thus, a positive (respectively negative) value corresponds to increased (respectively decreased) DNA damage between days 1 and 12 of exposure. The two black circles indicate the cases where a Kolmogorov–Smirnov test ($p = 0.05$) does not show the statistical difference of DNA damage (absence of a black circle means that a Kolmogorov–Smirnov test indicates the statistical difference of DNA damage, either positive or negative, between days 1 and 12 of exposure). L_A: leachate of soil A. L_B: leachate of soil B. P_{MSWI}: percolate of municipal solid waste incineration bottom ash.

days of exposure (Table 2b), P_{MSWIBA} was genotoxic with respect to each parameter at all concentrations and the effect was, once again, inversely dependent on the dose. Between days 1 and 12, a significant increase of DNA damage was observed in P_{MSWIBA} (Fig. 1). The response of larvae to exposure to P_{MSWIBA} was globally time-dependent for both parameters at all concentrations.

3.2. Bacterial genotoxicity

The Ames test (with or without S9 activation) did not reveal genotoxicity of any of the aqueous extracts (Table 3). Likewise, Mutatox gave no mutagenic response, except for L_A, where it indicated a positive mutagenic response without metabolic activation at a concentration of 2.9%.

Table 3
Results of the bacterial tests (Ames and Mutatox tests)

Assay	L_A		L_B		P_{MSWI}	
	+S9	−S9	+S9	−S9	+S9	−S9
Mutatox®	–	+ (2.9%)	–	–	–	–
Ames	–	–	–	–	–	–

'–' indicates that DW is non-mutagenic; '+' indicates a mutagenic activity. Value in percent is the lowest concentration providing a mutagenic effect.
L_A: leachate of soil A. L_B: leachate of soil B. P_{MSWI}: percolate of municipal solid waste incineration bottom Ash. +S9: in the presence of S9 mix; −S9: without S9 mix.

4. Discussion

The CA results often revealed significant background levels of DNA damage in negative controls, which were, furthermore, dependent on the time of exposure. Some authors (Guecheva et al., 2001; Singh et al., 1991) have demonstrated that DNA strand breaks in negative controls can be a consequence of cell isolation. However, in the present study, no prior cell isolation step was used, or required (contamination of peripheral blood erythrocytes is of the order of one lymphocyte per thousand erythrocytes). As previously suggested by Wilson et al. (1998), in agreement with Pacifici and Davies, 1991, aerobic organisms are constantly exposed to endogenous and exogenous oxygen radicals and related oxidants, and although anti-radical defense systems are present, a low level of oxygen radicals can induce oxidative stress in all cellular components, including DNA. Significant DNA damage in controls in some cell types may be related to DNA packaging and background alkali-labile sites, rather than to exogenous strand breaks (Mitchelmore and Chipman, 1998; Singh et al., 1989). Moreover, as suggested by Liepelt

et al. (1995), variable oxygen levels in test water can lead to variations in DNA integrity. This background in negative controls is to be expected in any biological test, such as, for example, the micronucleus test; our introduction of the evolution coefficient in the treatment of the results enables to consider the part of the DNA damage that was related to the treatment.

4.1. Soil leachates

The genotoxic potential of both soil leachates was clearly demonstrated, using the CA and the MNT. However, CA detects genotoxicity after as little as 24 h of exposure, earlier than the French Standard NF T 90-325 MNT (12 days, AFNOR, 2000) allows. Genotoxicity may be a function of the chemical composition of the leachates (Table 4). The genotoxic effects of L_A correlate well with those of Békaert (1999) in a previous study. This author simultaneous carried out the MNT, measurements of DNA adducts and EROD activity on *Xenopus* larvae reared in the leachate produced by the same lixiviation procedure. Both leachates contained a heavy load of contaminants (Table 4), L_A was contaminated to a large extent by metals and PCBs, and also by a fraction of PAHs and differents OCPs. The major contaminants of L_B were PAHs and, to a lesser extent, a metal fraction. It is well known that PCBs induce genotoxicity via production of reactive oxygen species (ROS) from metabolites (Sargent et al., 1989; Ludewig et al., 1998). The DNA-strand-breaking potential of OCPs has been widely demonstrated: for example, this effect has been shown for Trifluralin in human lymphocytes, using the CA (Ribas et al., 1995), for Heptachlor, Dieldrin and Aldrin on *Tradescantia*, using the MNT (Sandhu et al., 1989) and forhexachlorobenzene in rat hepatocytes, using the MNT (Canonero et al., 1997). PAHs can cause DNA damage in aquatic models either by direct DNA strand breakage or after being metabolized into reactive intermediates that form unstable DNA adducts (Nacci et al., 1992, 1996; Mitchelmore et al., 1998). Both processes are likely to trigger DNA excision repair mechanisms that give rise to DNA fragments detectable with the CA (Collins et al., 1997; Speit and Hartmann, 1995). Clastogenic and/or aneugenic abilities of several PAHs have also been established using the amphibian model (*X. laevis* and *Pleurodeles waltl*) as well as DNA adduct induction (Fernandez et al., 1989; Békaert, 1999). Heavy metals can bind to phosphates and a wide variety of organic molecules, including DNA base residues, and can lead to mutations by altering primary and secondary structures of the DNA (Wong, 1988). Genotoxic properties of Cu, Cd and Hg, have also been demonstrated on *X. laevis* and *P. waltl* (Mouchet, 2002) and similar results were obtained with Cr and Fe on *P. waltl* (Godet et al., 1996).

The leaching procedure approximates the natural contamination of the aquatic compartment and mimics transfer of micro-pollutants from the solid to the aquatic compartment. Its ecotoxicological relevance has already been highlighted in previous research on contaminated soils (Békaert, 1999; Békaert et al., 2002). This extraction procedure mimics the effects of strong rain-fall. Although only a small part of the initial toxic load was found in the corresponding leachate, the genotoxic results show that the low fraction of contaminants must be taken into account for the management of environmental risks.

With respect to the CA, a reduction of the level of DNA damage was observed between days 1 and 12 for both leachates. DNA damage even disappeared at the lowest concentration in the case of L_B. Reduction or disappearance of DNA damage has been already observed in previous studies on *Xenopus* exposed to some of the pure substances which were found in large amounts in the leachate of the soil B, including PAHs (Mouchet et al., 2005) and metals such as Cd, Cu and Hg, (Mouchet, 2002). Similarly, it was also observed on *Xenopus* reared in draining water from dredged sediments contaminated by PAHs and metals (Mouchet et al., in press). The presence of DNA crosslinks could explain the reduction in DNA damage level. Indeed, DNA crosslinks are known to result in retarded migration of DNA (especially in response to exposure to metals) under the alkaline conditions that we used for the CA (Pfuhler and Wolf, 1996; Merk and Speit, 1999). DNA–protein crosslinks are known to be formed by the metals, such as Cr, Ni and Cd, that were found in L_B. Moreover, a decrease (or disappearance) of DNA damage can stem from a variety of cellular processes, including DNA repair activities, detoxication processes and adaptative response to repeated doses. It is well established that repair processes are genetically controlled (Moustacchi, 2000).

Table 4
Chemical analysis of soils, soil leachates and percolate of the municipal solid waste incineration bottom ash

		$S_A^{(1)}$	$L_A^{(1)}$	$S_B^{(1)}$	$L_B^{(1)}$	$P_{MSWI}^{(2)}$
METALS ([1]: mg/kg D. Wt—[2]: mg/L)	Cd	20	<0.05	1	<.05	<0.005
	Cu	193	2.4	63	9.7	10
	Ni	28	1.1	37	5.2	0.052
	Pb	1266	4.5	251	76	0.116
	Zn	1954	16.2	1392	174	0.122
	Cr^{VI}	218	<0.05	57	8.3	0.21
	Hg	2	<0.001	0.3	<0.001	<0.001
	Fe	n.a.	n.a.	n.a.	n.a.	0.575
	As	n.a.	n.a.	n.a.	n.a.	<0.06
	Total of the 9 metals	3681	24.30	1801	273.25	11.08
PAHs ([1]: μg/kg D. Wt—[2]: μg/L)	Naphtalene	–	4.96	–	8.99	–
	Acenaphthylene	–	–	–	–	–
	Acenaphthene	174.33	–	–	2.27	–
	Fluorene	538.69	–	198.60	11.58	–
	Phenanthrene	2677.46	1.18	3447.98	92.32	–
	Anthracene	199.93	–	896.48	29.06	–
	Fluoranthene	1784.97	0.72	12,335.00	195.60	–
	Pyrene	1402.53	–	6723.57	157.12	–
	Benzo(a)anthracene	807.29	0.78	7024.42	131.20	–
	Chrysene	989.96	–	7027.80	120.32	–
	Benzo(b)fluoranthene	975.07	–	12,740.64	312.24	–
	Benzo(k)fluoranthene	950.89	–	4833.94	134.48	–
	Benzo(a)pyrene	400.30	0.85	7044.71	179.36	–
	Dibenzo(ah)anthracene	–	–	717.65	24.22	–
	Benzo(ghi)perylene	438.62	–	4201.81	154.24	–
	Indenopyrene	271.69	–	3009.89	102.40	–
	Total of the 16 PAHs	11,611.12	8.49	70,202.48	1655.41	–
PCBs ([1]: μg/kg D. Wt—[2]: μg/L)	PCB28	–	–	–	–	–
	PCB52	9437.87	1.96	–	–	–
	PCB101	13,409.23	2.84	–	–	–
	PCB118	13,612.72	4.15	–	–	–
	PCB153	7599.33	2.88	–	0.47	–
	PCB138	10,333.33	4.72	–	0.69	–
	PCB180	–	1.35	–	–	–
	Total of the 7 PCBs	44,954.61	17.9	–	1.16	–
OCPs ([1]: μg/kg D. Wt—[2]: μg/L)	Trifluralin	–	0.48	–	–	–
	Hexachlorobutadiene	–	–	–	–	–
	α-hexachlorocyclohexane	–	1.23	–	–	–
	Hexachlorobenzene	–	2.08	–	–	–
	β-hexachlorocyclohexane	–	2.20	–	–	–
	γ-hexachlorocyclohexane	82.68	–	–	–	–
	Di-hehachlorocyclohexane	–	0.65	–	–	–
	Alachlor	–	–	–	–	–
	Heptachlor	–	0.27	–	–	–
	Aldrin	–	0.78	–	–	–
	Isodrin	42.32	–	–	–	–
	Heptaepoxid	–	–	–	–	–
	o′-p′-Dichlorodiphenyldichloroethylene	–	–	–	–	–
	Aendosulfan	–	–	–	–	–
	p-p′-Dichlorodiphenyldichloroethylene	–	–	–	–	–
	Dieldrin	–	0.45	–	–	–
	o′-p′-Dichlorodiphenyldichloroethane	–	–	–	–	–
	Endrin	–	–	–	–	–

(continued on next page)

Table 4 (*continued*)

		$S_A^{(1)}$	$L_A^{(1)}$	$S_B^{(1)}$	$L_B^{(1)}$	$P_{MSWI}^{(2)}$
OCPs ([1]: μg/kg D. Wt—[2]: μg/L)	Bendosulfan	–	–	–	–	–
	p-*p*′-Dichlorodiphenyldichloroethane	–	–	–	–	–
	o′*p*′-Dichloro diphenyl trichloroethane	–	–	–	–	–
	p-*p*′-Dichloro diphenyl trichloroethane	–	–	–	–	–
	Total of the OCPs	125	8.14	–	–	–

S_A: soil A. S_B: soil B. L_A: leachate of soil A. L_B: leachate of soil B. P_{MSWI}: percolate of municipal solid waste incineration bottom ash. OCPs: organochlorinated pesticides. PAHs: polycyclic aromatic hydrocarbons. PCBs: polychlorinated biphenyls. n.a.: not analyzed. –: lower than the quantifiable limit. D. Wt: Dried weight.

Regardless of the nature of the DNA damage caused in *Xenopus*, an induced repair system may be effective or saturated by a genotoxicant. The repair system seems to be induced by a variety of factors: the concentration of genotoxicant, the nature of the DNA damage and its location on the DNA molecule could all play a part in controlling its induction. Each repair system preferentially acts on a particular kind of damage (Friedberg et al., 1995). Induction of a detoxication process implicating cytochrome *P*450 has been already demonstrated in *Xenopus* and *Pleurodeles* larvae after exposure to B[a]P (Békaert, 1999; Marty et al., 1989). The adaptative response could be a consequence of the induction of metallothionein by the daily exposure to metals; as suggested by Devi et al. (2001), from his work on the oral administration of Cd to mice) and by Fatur et al. (2002) (who exposed HepG2 cells to the metal). Such a response to early exposure to Cd would provide protection from the effects of the metal during subsequent treatment and hence a reduction in DNA damage.

4.2. MSWIBA percolate

The genotoxic potential of P_{MSWIBA} was clearly established by the CA from the first day of exposure, whereas both the CA and the MNT produced positive results after a 12-day period, at all concentrations tested. Genotoxic behaviour is largely due to metals, which are the exclusive contaminants of P_{MSWIBA} (Table 4). Close correlations between toxic or genotoxic potential of complex mixtures and pure substances which are present in the mixture are hard to establish. Nevertheless, it should be emphasised that the Cu concentration in the percolate was 400 times higher than the concentration that induces micronuclei and 2000 times greater than the concentration that was shown to cause DNA damage in a previous study (Mouchet, 2002). Finally, the Hg concentration in the percolate was similar to that producing DNA damage in *Xenopus.*

According to the CA, genotoxicity increased with the time of exposure (from 1 to 12 days). Even if phenomena that were previously observed in soil leachates also occur in P_{MSWIBA}, it is likely that other events, (its antagonistic or synergistic effects), also take place. Increased genotoxicity could probably be explained by saturation or inactivity of the repair system. Indeed, precise conditions are required to control genetically the induction of the repair system: these include genotoxicant concentration and/or nature of the DNA damage and/or its location on the DNA molecule. In addition, bioaccumulation of metals by aquatic organisms is well documented (Kock and Van Het Groenewoud, 1985; Flessas et al., 2000; Zyadah and Abdel-Baky, 2000). For example, bioaccumulation of Hg (by a factor of 1000) has been demonstrated in *P. waltl* and *X. laevis* after 12 days of exposure (Zoll et al., 1988; Zoll-Moreux, 1991). This phenomenon could contribute to the increase of DNA damage observed between days 1 and 12 in the present work.

P_{MSWIBA} was obtained by flowing reconstituted rain-water through lab lysimeters, under gravity. This aqueous extraction mimics natural phenomena of rain-water seepage during storage under conditions of exposure to bad weather. Indeed, percolation by natural gravity reproduces the vertical infiltration of contaminants into ground water. Thus, our genotoxic results show that these conditions can produce a genotoxic impact on the environment. Streaming rain-water and/or level variations of underground water can load water with metals. Despite this, P_{MSWIBA} has

been classified as valorizable for the construction of roads and similar structures, such as embankments, underlays or foundations of roads or car parks.

Numerous studies on the environmental impact of MSWIBA, prepared by different kinds of extraction procedure, such as laboratory lysimeter (Bruder-Hubscher et al., 2001a), natural percolation (Bruder-Hubscher et al., 2001b), or various others methods (Van der Sloot et al., 2001; Zhang et al., 2002) have focussed on characterising the physico-chemical nature of pollutants discharged into the environment. In contrast, to our knowledge, studies aiming at an evaluation of the genotoxic impact are rare and most of these are based solely on bacterial tests (Ames: Silkowski et al., 1992; Ames and Microtox: Filipic, 1995).

In conclusion, MSWIBA management implies a serious risk evaluation that is not exclusively based on physico-chemical characteristics as provided by ministerial circulars. MSWIBA management should much better take into account the in vivo genotoxic risks to the aquatic compartment.

4.3. Comparison of the results of the biological tests

4.3.1. CA versus MNT

The micronucleus test and the comet assay are sensitive tools for an effective evaluation of genotoxicity biomarkers in aqueous extracts, such as soil leachates and MSWIBA percolates. The genotoxic potential of P_{MSWIBA} and both leachates was clearly established by the CA from the first day of exposure onward. It is in agreement with the delay of genotoxic responses observed in a previous study with the CA using *Xenopus* exposed to draining water from dredged sediments (Mouchet et al., in press). The comet assay can directly detect DNA strand breaks, which may be induced very early after exposure to pollutants. Micronuclei are the result of chromosome breaks (and/or mitotic anomalies) that necessitate a passage through mitosis before they can be recognised. Thus, the use of the CA on *Xenopus* larvae could be suggested as an initial screening test (as also suggested by Moller et al., 2000), since it detects genotoxicity from 24 h of exposure i.e., earlier than the French Standard NF T 90-325 Micronucleus test specifies (12 days; AFNOR, 2000). Then, the MNT could be used in a second step (Table 5).

Table 5
Comparison of the genotoxicity results (comet assay, micronucleus test, Ames test and Mutatox)

ASSAYS	Comet		Micronucleus	Ames		Mutatox	
				−S9	+S9	−S9	+S9
	1 day	12 days	12 days			–	–
L_A	*1.5%	*3%	*3%	–	–	*2.9%	–
L_B	*6%	*25%	*6%	–	–	–	–
P_{MSWI}	*3%	*3%	*3%	–	–	–	–

L_A: leachate of soil A. L_B: leachate of soil B. P_{MSWI}: percolate of municipal solid waste incineration bottom ash. Values in percentage correspond to the weakest effective concentration of aqueous extracts in the middle. *: Genotoxicity; –: no genotoxicity.

The sensitivity of the CA is often considered as being greater than that of the MNT (He et al., 2000). The difference of sensitivity between these two tests is generally explained by their different endpoints. In this study, the sensitivity of CA after 1 day of exposure and that of MNT after 12 days of exposure were similar for L_B and P_{MSWIBA}. In contrast, the sensitivity of CA (1 day) was higher than that of the MNT (12 days) in the case of L_A. Indeed, the lowest effective concentration of L_A with the MNT (3%) was higher than with the CA (1.5%). Contaminants of L_A can induce DNA strand breaks (single–double strand breaks, alkali-labile sites, uncompleted excision-repair-sites), whereas the MNT only detects micronuclei that result from chromatid or chromosomal fragments. The concentration of contaminants in 1.5% L_A may induce primary DNA damage, that may not lead to micronuclei formation. Therefore, using *Xenopus*, the CA detects genotoxic potential of L_A at lower concentrations than the MNT.

At the end of treatment (12 days) the sensitivity of the CA and the MNT was similar for L_A and P_{MSWIBA}. In contrast, in the case of the L_B, sensitivity of the CA was lower than that of the MNT. Indeed, the weakest effective concentration of L_B with the MNT (6%) was lower than that with the CA (25%).

4.3.2. Amphibian tests versus bacterial assays

The genotoxicity of the three aqueous extracts was detected with the CA and with the MNT (Table 5). In contrast, none of them was found to be genotoxic using the Ames test or the Mutatox test (L_B and P_{MSWIBA}). Only L_A was identified as genotoxic both by amphibian tests and the bacterial Mutatox test. It is

generally agreed that the filtration (0.45 μm) used for the technical Ames test procedure is one of the causes of the lower sensitivity of this bioassay compared to the other in vivo tests such as the MNT (Békaert, 1999); filtration partly eliminates the contaminant fraction which is adsorbed on the solid matter in suspension which is available for amphibian larvae. However, this argument does not hold for the Mutatox test, in which no preliminary filtration (0.45 μm) is carried out. Our results imply that the in vivo tests of genotoxicity are necessarily complementary to the in vitro tests for evaluating the genotoxic potential of environmental media. Likewise, in a previous study (Mouchet et al., in press), the sensitivity of the CA on *Xenopus* exposed for 1 day to five draining waters from dredged sediments was also greater than the sensitivity of bacterial tests (Ames and Mutatox) in four cases out of five.

5. Conclusion

The micronucleus test and the comet assay are sensitive tools for the effective evaluation of genotoxicity biomarkers. In spite of the low contaminant load found in the extremely high dilutions of P_{MSWIBA} and soil leachates (potentially corresponding to environmental concentrations), genotoxic effects on amphibian larvae were revealed both by the CA and the MNT. Contaminated soils and the MSWIBA each constitute an important reservoir of contaminants for the aquatic environment. Once again, we confirm the ecotoxicological relevance of *Xenopus* larvae in biomonotoring studies. In contrast, no genotoxic response was obtained with bacterial tests (except for one leachate in the case of Mutatox). Consequently, management of contaminated matrices like MSWIBA and soils requires in vivo genotoxic evaluation such as that provided by the CA and MNT on *Xenopus*.

We suggest using the CA on *Xenopus* larvae for screening purposes, since it can detect genotoxicity after as little as 24 h of exposure, i.e., earlier than the period specified by French Standard NF T 90-325 Micronucleus test (12 days; AFNOR, 2000). The MNT could be used subsequently, for confirmation. In any event, as we concluded in a previous study on draining water from dreged sediments (Mouchet et al., in press), MNT and CA provide complementary information, depending on the kind of matrice. Since the MNT detects chromosome/genome mutations and the CA detects DNA primary damage (including repairable DNA damage, the combined use of both tests on the same biological model is recommendable for genotoxicity testing. The complexity of deleterious effects on an aquatic vertebrate such as *Xenopus* could then be better taken into account.

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