Use of a battery of bioassays to classify hazardous wastes and evaluate their impact in the aquatic environment

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This case study can be used in conjunction with *Module III Toxic Contaminants in the Aquatic Environment, Section 4 Monitoring of toxicants in aquatic ecosystems* of the NEAR curriculum. It demonstrates how bioassay approaches can be used to evaluate the toxicity of solid wastes and leachates as a means of predicting their ecotoxicity in the aquatic environment.

1 Introduction

In today's world, generation, storage, treatment, transport, recovery, transboundary movement, and disposal of wastes pose formidable problems for society and represent a serious threat for human health and the environment. Great concern exists for the future if this issue is not properly addressed (Rummel-Bulska, 1993). Consequently, it is advisable to manage waste as well as possible, which implies an adaptation of the legislation and innovations from the technological and scientific point of view.

Waste management is now moving from a "ways of dealing-based approach" (disposal, incineration and/or treatment of wastes) to an "objectives-based approach" (reduction, re-use, valorisation, stabilization/solidification, vitrification, risk assessment, ecocompatibility…). In Agenda 21 (i.e. http://www.un.org/esa/sustdev/documents/agenda21/index.htm), for example, the framework for required action is based on a hierarchy of objectives and focused on the four following major waste-related program areas:

- Minimizing wastes;
- Maximizing environmentally sound waste reuse and recycling;
- Promoting environmentally sound waste disposal and treatment;
- Extending waste service coverage.

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One of the challenges to achieving such an "objectives-based approach" is improvement of waste classification methodologies. Managers need a sound regulatory framework and to dispose of the adapted technological methods that have been used in prospective studies for establishing the optimum approach to waste management. In this context, the present case study gives an example of how ecotoxicological assessment of wastes contributes to hazardous waste classification under regulatory requirements. Moreover, an example of environmental hazardous assessment of waste deposits illustrates the potential of such a waste management procedure.

2 Hazardous waste legislation: Where are we now?

In the European Union (EU), the overall structure for the framework for waste management is set out in a series of directives, decisions, regulations and resolutions on waste and hazardous waste (i.e. http://europa.eu.int/eur-lex/en/lif/reg/en_register_15103030.html). Among the existing rules, Council Directive 91/156/EEC (CD, 1991a), amending the Council Directive 75/442/EEC (CD, 1975) often referred to as the Framework Directive on Waste (FDW), constitutes the legal framework for the avoidance, environmentally sound management and disposal of all wastes (Slide 3). This directive also defines wastes as "*any substance or object in the categories set out in Annex I which the holder discards or intends or is required to discard*". In other words everything that belongs to any of the 16 categories of waste outlined in Annex I of this directive is regarded as waste.

In connection with the FDW, Council Directive 91/689/EEC (CD, 1991b), referred to as the Hazardous Waste Directive (HWD), aims to identify which waste must be regarded as hazardous and to ensure the correct management and regulation of such waste. For the purposes of the directive, hazardous wastes are defined in essence as:

- Wastes displaying one or more of the 14 hazardous properties listed under H1 to H14 in Annex III of the directive (see Table 1, Slide 4); these 14 criteria are distributed among 4 types: H1 to H3 = physical hazard; H4 to H12 = hazard for human health; H13 = hazard following elimination of waste; H14 = environmental hazard.
- Wastes containing any constituents listed in Annex II of the directive and having one or more hazardous properties. The list goes from C1 to C51. For example, C25 is asbestos.

The hazardous character of a waste is thus defined by reference to a list of properties (physico-chemical, toxicological and ecotoxicological), or by reference to its composition. As a result, a waste list, the so-called European Waste Catalogue (EWC) was established in Decision 94/3/EC pursuant to the FDW and a subset of this, the Hazardous Waste List (HWL), was introduced under Decision 94/904/EC pursuant to the HWD. However, since 1 January 2002, the two decisions have been replaced by Decision 2000/532/EC which envisaged the amalgamation of the EWC and the HWL into a single list by indicating on the EWC/HWL if a waste is hazardous.

Table 1 The properties of hazardous waste according to Council Directive 91/689/EEC

H1	**Explosive**
H2	Oxidizing
H3A	Highly flammable
H3B	Flammable
H4	Irritant
H5	Harmful
H6	Toxic
H7	Carcinogenic
H8	Corrosive
H9	Infectious
H10	Teratogenic/toxic for reproduction
H11	Mutagenic
H12	Substances and preparations which release toxic or very toxic gases in contact with water, air or an acid
H13	Substances and preparations capable by means, after disposal, of yielding another substance, e.g. leachate, which possesses any of the characteristics listed above
H14	Ecotoxic

Source: CD, 1991b

3 Assessment of the H14 "Ecotoxic" criterion: the French conceptual methodology

Determination of the hazardous characteristics corresponding to the various H criteria in Table 1 is based on test methods. While some of the methods developed for risk assessment of chemicals may be directly applied, there is no specific method immediately lending itself to application to criterion H14 (i.e. "Ecotoxic"). To fill the gap, the French Ministry of Environment (1998) proposed a working document under the title "*Criteria and methods for the assessment of the ecotoxicity of wastes*". This methodology was developed with the aim of it being accepted by the EU as a technical support procedure for waste classification under criterion H14.

The procedure described in the French proposal follows a general scheme as summarized in the Fig. 1 (Slide 5). Globally, the ecotoxicity of any waste can be assessed through either its chemical composition or its ecotoxicological characteristics by applying a battery of bioassays. Both approaches can be used on raw waste and on its leachate prepared in well-defined conditions. The chemical composition is used as a positive criterion, i.e. the presence of at least one pollutant in a concentration higher than the limits fixed in the proposal (Table 2) allows classification as ecotoxic and, consequently, as hazardous under the terms of the HWD. If the chemical characterization is inconclusive, ecotoxicological characterization is needed. The proposal assumes that the ecotoxicological characterization can be used as a positive or a negative criterion. The positive criterion means that if at least one bioassay shows a toxicity value (i.e. an effective concentration expressed in percentage of dilution of either liquid extract or solid waste) inferior to a limit value (see Table 3) the waste is classified as hazardous. On the contrary, the negative criterion presumes that the waste can be classified as non-ecotoxic if the bioassay toxicity values are higher than the fixed limits.

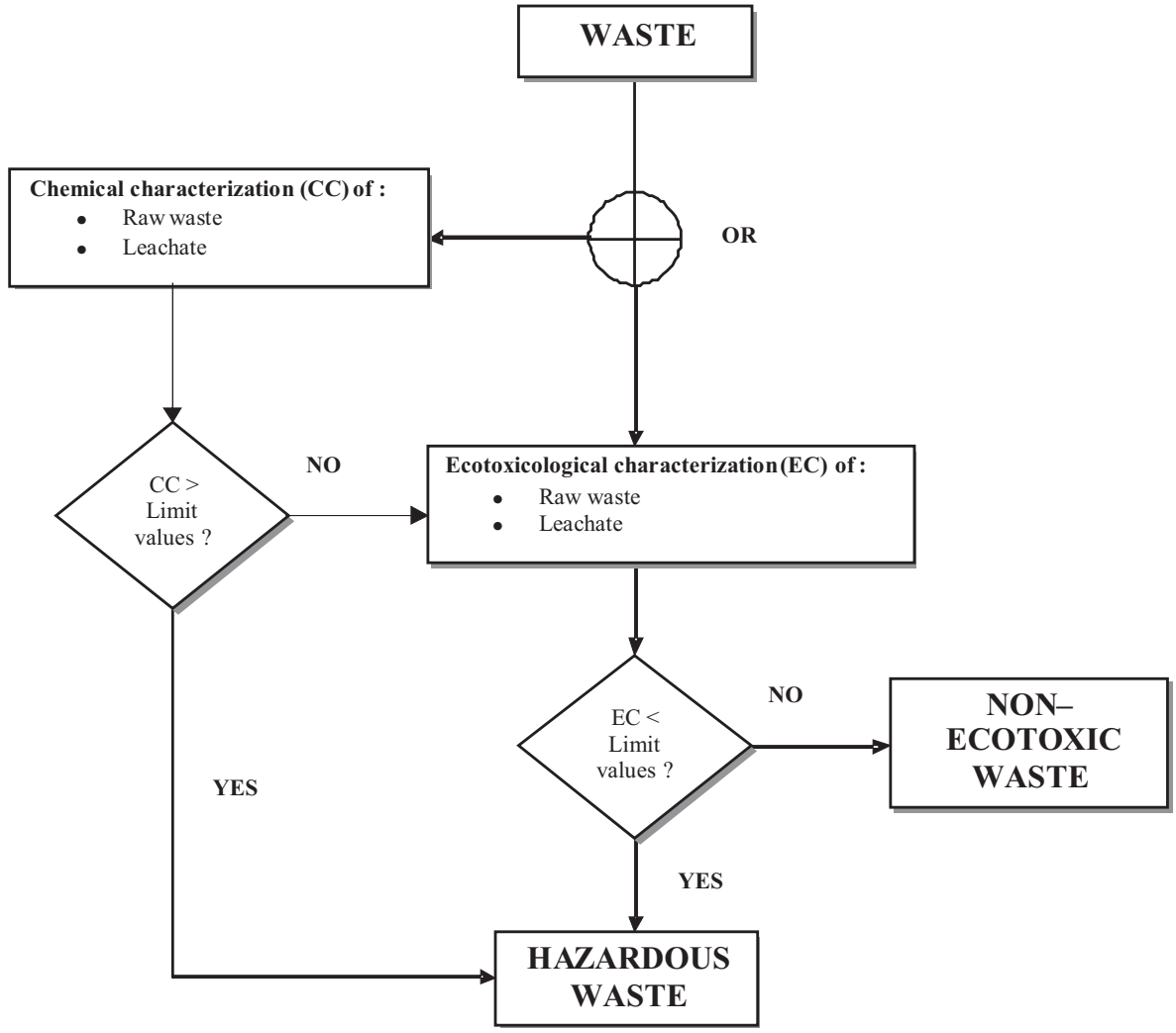

Figure 1 Criteria and methods for the assessment of the ecotoxicity of wastes (After: French Ministry of Environment, 1998)

Table 2 Chemical limits defined in the French proposal for "criteria and methods for the assessment of the ecotoxicity of wastes" CMAEW

Parameter	CMAEW (mg l^{-1})
Phenol index	0.1
Total hydrocarbons	10
Arsenic	0.05
Cadmium	0.2
Chromium	0.5
Chromium (VI)	0.1
Copper	0.5
Lead	0.5
Mercury	0.05
Nickel	0.5
Zinc	2

Source: French Ministry of Environment, 1998

Table 3 Ecotoxicological limits defined in the French proposal for "criteria and methods for the assessment of the ecotoxicity of wastes" CMAEW

	Biological indicator	Limit value[a]
Indirect measure of toxicity (leachate)	Microtox™ - EC_{50}[b] (30 min exposure)	10%
	Daphnia magna – EC_{50} (48h exposure)	10%
	Pseudokirchneriella subcapitata – EC_{20} (72h exposure)	0.1%
	Ceriodaphnia dubia – EC_{20} (7d exposure)	0.1%
Direct measure of toxicity (solid–phase)	Plant – EC_{50} (14 d exposure)	10%
	Worm – EC_{50} (14 d exposure)	10%

[a] Minimum effective concentration limits for non-ecotoxic wastes; [b] Effective concentration, i.e. concentrations producing an effect on 50% (or 20%) of the population
Source: French Ministry of Environment, 1998

The current procedure was applied in this case study involving two kinds of solid waste, a municipal solid waste incinerator bottom ash BA (EWC/HWL code 19 01 01) and a slag from a second smelting of lead 2SL (EWC/HWL code 10 04 01). Both wastes are granular wastes with a high content of metals and salts.

4 Implementation of the French methodology

4.1 Pretreatment and preservation of the samples of wastes (Slide 6)

Within a period of 4 days after arrival at the laboratory, the samples of BA were submitted to a crushing procedure with the aim of obtaining fragmented material with a particle size lower than 4 mm, as required by the leaching procedure. Because the samples of 2SL corresponded to a powder in which the particle size was lower than 4 mm, no crushing treatment was applied. For both wastes, the moisture content was determined by drying a small portion of each samples at 105 ± 5 °C, until constant weight was reached. The values obtained were then taken into account for the adjustment of the liquid to solid ratio (L/S) in the leaching procedure. All samples of BA and 2SL were stored at ambient temperature inside tightly sealed containers to prevent contact with the atmosphere prior their use for experiments.

4.2 Batch leaching procedure

The pre-treated samples were submitted to the leaching methodology described in the draft European standard EN 12457 – 2 (2002) using a liquid to solid ratio (L/S) of 10:1 (Fig. 2, Slide 7). Briefly, sub-samples prepared from each sample were brought into contact with deionised water in the defined L/S ratio for 24 hours with agitation at constant temperature of 20 ± 2 °C. The different mixtures were placed in capped one-litre polyethylene bottles and the extraction process was performed in a roller-rotating device working at 100 rpm. After 24 hours of agitation, each mixture was settled for 15 minutes and centrifuged for 10 minutes at 3,500 rpm in order to

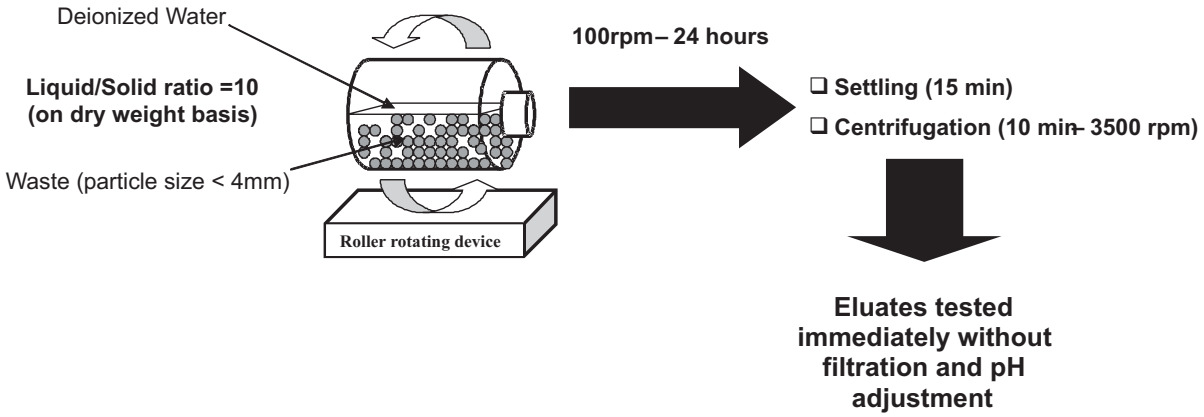

Figure 2 Leaching methodology according to the European standard EN 12457 (2002) part 2 using a liquid to solid (L/S) ratio of 10:1

remove the suspended matter from the leachates. The ecotoxic potential of the obtained supernatants was then assessed immediately without filtration and pH adjustment.

4.3 Ecotoxicological characterization of leachates (Slide 8)

The ecotoxicological parameters analyzed in the leachates were obtained from acute bioassays (i.e. Microtox™ test and *Daphnia magna* immobilization test) and from chronic bioassays (i.e. algal growth inhibition test and *Ceriodaphnia dubia* reproduction inhibition test). In addition, the direct ecotoxicity of the pre-treated wastes was assessed using the acute plant growth inhibition test.

4.3.1 Microtox™ test

The Microtox™ test measures the decrease in bioluminescence induced by depletion in the cell metabolism due to a toxic effect of the tested leachate. Each test was performed according to the procedure described in the standard AFNOR T90-320-3 (1999). Briefly, the test system used the special light emitting bacterial reagent (*Vibrio fischeri*) supplied in freeze-dried form by Azur Environmental (Carlsbad, CA, USA), culture medium (NaCl 2 per cent) and the Microtox™ temperature-controlled photometer (Microtox™ Model 5000). In each test, different dilutions of tested leachate were compared with one control. The freeze-dried bacterial culture was initially reconstituted with deionised water (Milli-Q water purification system) at 4 °C. In each of cuvettes, the light produced by 0.1 ml of a bacterial suspension prepared by mixing 10 µl of the bacterial culture and 90 µl of NaCl 2 per cent solution and equilibrated to 15 °C was measured. Then, 0.9 ml of the test leachate dilutions adjusted to 2 per cent NaCl and 0.9 ml of 2 per cent NaCl control solution were added to the respective cuvettes. Light output from each cuvette was measured after 30 minutes. Results were corrected by time-dependent change in light emission under test conditions without any toxic influence and the percent difference between initial light output and final light output was quantified.

4.3.2 Algal growth inhibition test

The algal growth inhibition test is based on the measurement of growth inhibition of the algae *Selenastrum capricornutum* (strain ATCC 22662, Rockville, MD, USA), renamed *Pseudokirchneriella subcapitata*, during 72 hours of exposure. Each test was performed according to the adapted procedure proposed in the standard AFNOR T90-375 (1998) using sterile 96-well microplates as described earlier by Blaise *et al*. (1986). Briefly, inocula from cultures in the mid-exponential phase were adjusted to 40,000 cells per ml in the standard growth medium at double strength, and 125 µl was micropipetted into each of the 60 internal microplate wells. Then, 125 µl of leachate solution at twice the desired concentration was added (water was used for controls). Each microplate was sealed with its cover to minimize evaporation during the exposure period. After 72 h of incubation under continuous illumination of 3,000 lux at 24 ± 1 °C, the algal growth was followed by measuring the fluorescence using a microplate fluorimeter. The fluorimeter was set with the excitation filter at 440 nm and emission filter at 640 nm.

4.3.3 Daphnia magna *immobilization test*

The *Daphnia magna* immobilization test is based on the measurement of mobility inhibition of this cladoceran during two days of exposure. It was conducted according to the standard AFNOR T90-301 (1996). Each assay was carried out in 28 glass cups, which allowed testing of six concentrations and one control, with four replicates. Ten millilitres of the tested leachate dilution prepared in the standard dilution medium (dilution medium alone acted as the control) were introduced in each cup. No food was added. The test was initiated by transferring five daphnids which were less than 24 hours old into each cup. Next, all cups were covered to reduce evaporation and then incubated for 48 h at 20 ± 1 °C in a temperature controlled chamber in darkness. After 24 h and 48 h of incubation, the numbers of motile and/or immobilized daphnids were counted.

4.3.4 Ceriodaphnia dubia *reproduction test*

The *Ceriodaphnia dubia* reproduction test is based on the measurement of reproduction inhibition of this cladoceran after seven days of exposure. It was conducted according to the standard EPA 600/4–91/002 (1994). Test animals were exposed for seven days in a daily static-renewal system to different test solutions (one control and five concentrations, with ten replicates) at 25 ± 1 °C in a temperature-controlled chamber with 16h:8h light:dark photoperiod. Illumination ranged from 300 to 500 lux. Prior to the start or the renewal of the test, a stock of 500 ml for each of the five test solutions was prepared by mixing the appropriate volume of leachate to obtain the desired dilution, 10.5×10^7 cells of green algae *Pseudokirchneriella subcapitata*, 1.15 ml of a 5 g l^{-1} fish food suspension (Tetramin®), 1.15 ml of a 5 g l^{-1} dried cereal leaves suspension (Cerophyll®) and growth medium to adjust to the final volume. At the same time, a stock of 500 ml of control solution was prepared by mixing only the nutritive mixture and the growth medium. The growth medium corresponded to a mixture of two French commercial natural waters,

Evian and Volvic respectively with a 1:4 v/v proportion. Then, test and control solutions were divided up to give 20 ml each test cup. At the start of each test, organisms less than 24 h old and all those within 6 h of the same age were individually placed in the test cups. For each daily renewal of the test and control solutions, the organisms were transferred from the old cups into new cups containing test and control solutions freshly prepared. During the seven days of exposure, mortality and reproduction of each animal were recorded at each daily renewal of medium.

4.3.5 Plant growth inhibition test (Slide 9)

The phytotoxicity test is based on the measurement of growth inhibition of the lettuce (*Lactuca sativa*) after 14 days of exposure. It was conducted according to the standard OECD 208 (1984). The reference soil was a natural loamy soil, and samples were taken from the 0–25 cm surface layer (organic matter 3 per cent, pH 6.6). Each sample of pre-treated waste was incorporated into the soil at different concentrations prior to potting. Three pots (disposable plastic, 7 cm diameter, 6 cm height) were used for each test concentration and 15 lettuce seeds were sown in each pot containing 100 g of soil or a mixture of soil and solid waste. Water evaporation was determined by daily weighing of pots and any loss was compensated by addition of distilled water. Plants were grown at 24 ± 1°C in a temperature-controlled chamber with lighting of 1,600 lux under a 16h:8h light:dark photoperiod. Fourteen days after planting, the total number of germinated plants was recorded, and then lettuces were cut at soil level in order to determine the wet and dry mass of the plant material.

4.3.6 Statistical analyses

For each test, results were expressed in terms of effective concentrations EC_{50} or EC_{20} (i.e. concentrations that cause respectively 50 per cent and 20 per cent of effect on the assessment endpoint). For the algal, ceriodaphnid and plant tests, EC_{20} or EC_{50} were determined by regression using the ICp method based on a linear interpolation of means. Daphnid EC_{50} and Microtox™ EC50 were determined by regression using a Probit model and a log–linear model respectively. Calculations were performed using the commercial software package TOXSTAT which can be purchased from Western Ecosystem Technology Inc. (address via http://www.west-inc.com).

5 Classification of the tested solid wastes according to the French methodology

Table 4 summarizes the ecotoxicological results obtained for each waste. The range of reported EC_{50} endpoints obtained from BA leachates (Slides 10 and 11) varied from 26.30 per cent (Microtox™ test) down to 2.12 per cent (algal test), whereas it varied from 0.43 per cent (algal test) down to 0.07 per cent (Microtox™ test) for the 2SL leachates (Slides 12 and 13). Biomass and germination results in the lettuce growth tests clearly demonstrated that some effects were also detected with the solid–phase procedure and that the 2SL displayed a higher toxicity than

Table 4 Direct and indirect ecotoxicity of a municipal solid waste incinerator bottom ash (BA) and a slag from a second smelting of lead (2SL): comparison with limits defined in the French proposal for the H14 criterion assessment

	Measurement endpoints	BA	2SL	Limit values
INDIRECT TEST		Leachate (L/S=10): Results in % of leachate		
Microtox™ (30 min)	EC_{50}	26.30	0.07*	10
D. magna (48h)	EC_{50}	3.70*	0.40*	10
P. subcapitata (72h)	EC_{20}	0.88	0.19	0.1
	EC_{50}	2.12	0.43	/
C. dubia (7d)	EC_{20}	20.40	0.20	0.1
	EC_{50}	22.70	0.28	/
DIRECT TEST		Solid waste: Results in % of dry waste equivalent		
Lactuca sativa (14d)	EC_{50} (germination)	40.00	1.20*	10
	EC_{50} (fresh biomass)	27.80	1.60*	10
	EC_{50} (dry biomass)	31.25	1.70*	10

* – Effective concentration (EC) inferior to the minimum limit value authorized in the methodology (i.e. Fig. 1 and Table 3): in this case, the waste is considered as hazardous.
Source: French Ministry of Environment, 1998

the BA. Such results obtained by direct and indirect measures of toxicity point out that the 2SL possesses a higher level of hazard potential than the BA.

Although the various bioassay measurement endpoints clearly do not have the same ecotoxicological significance (e.g. reproduction EC_{50} vs mortality EC_{50}), they nevertheless allow ranking each waste as a function of their sensitivity. For the BA leachate, the sequence in decreasing order of sensitivity was as follows: algal test > daphnid test > Microtox™ test > ceriodaphnid test. This information clearly identifies the algal test as a good candidate to assess BA toxicity leachate fluxes. The sensitivity of algae for this type of waste had previously been shown by Lambolez *et al*. (1994), Ferrari *et al*. (1999) and Lapa *et al*. (2002). In contrast, the decreasing sensitivity sequence for the 2SL leachates was as follows: Microtox™ test > ceriodaphnid test > algal test ≈ daphnid test. This indicates that the Microtox™ test should be a good indicator of the 2SL toxicity leachate fluxes. However, because a recent literature review demonstrated a complete lack of ecotoxicological data for this type of slag, no comparison could be made with other results.

Considering the limit values defined in the French proposal for the H14 criterion assessment, it is clear that the 2SL can be classified as hazardous waste because three of the five applied bioassays showed toxicity values inferior to their corresponding fixed limits. Such a classification is in accordance with the current EWC/HWL (cf. §2) which identifies this kind of residue as hazardous. For the BA, although only the acute daphnid test displayed a toxicity value lower than the recommended limit, it is sufficient to classify this waste as hazardous (Slide 14). However, in contrast with the 2SL classification, the current EWC/HWL does not clearly identify this kind of waste as hazardous. Even if some other studies have also pointed out the hazardous character of BA (Lapa *et al*., 2002), the difficulty of classifying them as such in the EWC/HWL may be linked to the large variability in the responses to the test methods because of

their variability in composition, in time and location (Radetski *et al*., 2004). Such results show also that the most sensitive test (i.e. algal test for the BA) may not be the test that enables classifying a waste as hazardous according the French methodology, which underlines the importance of using a battery of bioassays.

6 Using the H14 criterion assessment for environmental waste management

Waste classification under the criterion H14 may be taken further and used to highlight the potential hazardous impact of waste leachates on aquatic biota and thus, to ensure that unacceptable adverse effects would not arise from storage, treatment, re-use or disposal of the waste. In other words, this approach may be used as a prerequisite step to select the most suitable way for managing a waste. For example, because the ecotoxic hazard potential of the BA and the 2SL is indicated, these two wastes need to be either evaluated in a higher tier of risk assessment or treated before their valorisation, or stored under specific conditions (e.g. in a special landfill). The importance of this for aquatic biota is evident when considering that deposition of these two wastes directly in the environment, without the precaution of storage, has generated ecotoxic percolates. Indeed, two large field-scale leaching tests were built on an experimental site to simulate real conditions of a waste deposit receiving rain or run-off water and located near a river receiving effluents after percolation through the waste (Perrodin *et al*., 2002) (Slide 16). The first field leaching test consisted of a large tank where 39 tons of BA received water leading to the production of 2 m^3 of percolates per ton of dry BA every four months. The second one consisted of a smaller tank than the previous where 0.45 tons of 2SL received water leading to the production of 7.5 m^3 of percolates per ton of dry 2SL every four months. On the whole, three fractions (P) from each tank were recovered *in situ*. These fractions were defined as follows:

- P0.5, P1 and P2 corresponded to the cumulated quantities of percolates, according to the L/S ratios of 0.5, 1 and 2 for the BA (expressed in the cumulated volume of the leachate obtained at the exit of the tank by the dry-weight of waste),
- P2, P2.5 and P7.5 corresponded to cumulated quantities of percolates, according to the L/S ratios of 2.5, 5 and 7.5 for the 2SL.

When received at the laboratory (48 h after being sent from the field), the samples were settled for 15 minutes and centrifuged for 10 minutes at 3,500 rpm. Then, the ecotoxicity of the different fractions was assayed using the same battery of aquatic bioassays presented in Table 4 (i.e. Microtox™, algal, daphnid and ceriodaphnid tests).

Figure 3 summarizes the ecotoxicological results obtained for each waste and each cumulated percolate recovered *in situ*. Whatever the waste and the bioassays, the reported EC_{50} indicated: i) that BA percolates (Fig. 3a, Slide 17) appeared to be less hazardous than 2SL percolates (Fig. 3b), ii) that the first fraction is the most ecotoxic, and iii) that the ecotoxicity of the percolates was reduced as a function of the L/S ratio reached. However, at the end of the experiments, no threshold “without apparent ecotoxic effects” was reached for both wastes because

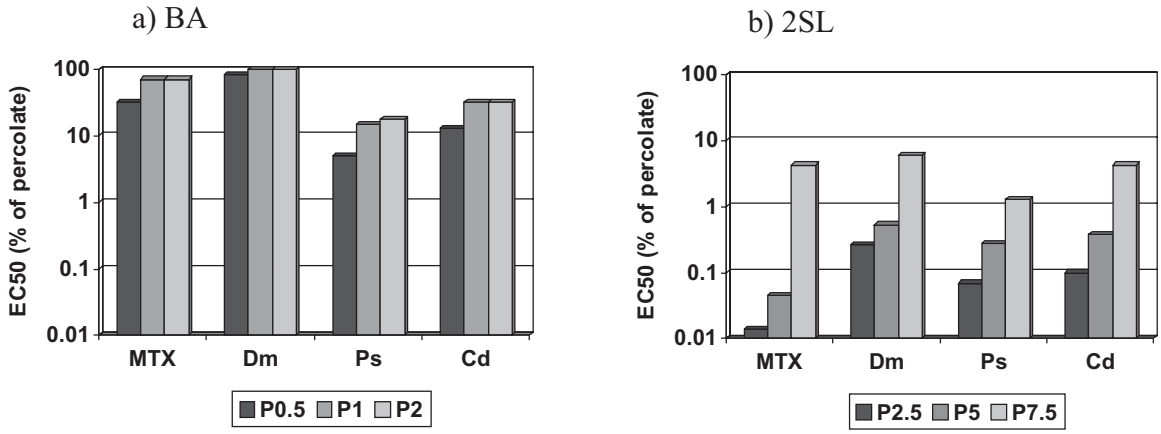

Figure 3 Ecotoxicity of cumulated percolates of a) a municipal solid waste incinerator bottom ash (BA) and b) a slag from second smelting of lead (2SL) from field experiments (MTX = Microtox; Dm = *Daphnia magna*; Ps = *Pseudokirchneriella subcapitata*; Cd = *Ceriodaphnia dubia*)

residual ecotoxicity was observed in the last fractions (between 10 and 100 per cent for BA and 1 and 10 per cent for 2SL). Consequently, the long-term ecotoxic potential hazard of these waste percolates for aquatic biota must be assumed.

This conclusion is reinforced by the ecological approach presented by Perrodin *et al*. (2002). Briefly, outdoor artificial streams (5 m, 440 litres, 1 control + 3 concentrations) colonized by aquatic invertebrate communities were supplied continuously by water which had received the same effluents produced by the field-scale leaching tests but having previously percolated through permeable subsoil. In these conditions, it was shown that a 10 per cent concentration of BA percolates was sufficient to produce significant effects on the abundance, richness and emergence of the organisms whereas only a 1 per cent concentration of 2SL percolates was needed (except for emergence, on which no effect was observed because of the season) (Slides 18 and 19).

7 Conclusion

The contribution of the ecotoxicological approach to hazardous waste classification under the regulatory requirement has been shown. It is evident that the use of bioassays to evaluate the toxicity of wastes is strongly recommended in order to have a more direct and integrated estimate of their environmental toxicity (Lambolez *et al*., 1994; Ferrari *et al*., 1999). In this sense, a preliminary assessment of the potential hazard of waste, as part of the potential impact studies, may be used as a prerequisite step to select the most suitable way for managing a waste in order to avoid possible surface and groundwater contamination. Moreover, coupling ecotoxicological and ecological approaches will provide even greater understanding during prospective or retrospective waste impact studies on the aquatic environment.

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