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ScienceDirect
Journal of Hazardous Materials 146 (2007) 227–236
Journal of Hazardous Materials
www.elsevier.com/locate/jhazmat

Evaluation of meat and bone meal combustion residue as lead immobilizing material for in situ remediation of polluted aqueous solutions and soils: "Chemical and ecotoxicological studies"

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Received 22 September 2006; received in revised form 1 December 2006; accepted 6 December 2006
Available online 15 December 2006

Abstract

As a result of bovine spongiform encephalopathy (BSE) crisis, meat and bone meal (MBM) production can no longer be used to feed cattle and must be safely disposed of or transformed. MBM specific incineration remains an alternative that could offer the opportunity to achieve both thermal valorization and solid waste recovery as ashes are calcium phosphate-rich material. The aim of this work is to evaluate ashes efficiency for in situ remediation of lead-contaminated aqueous solutions and soils, and to assess the bioavailability of lead using two biological models, amphibian *Xenopus laevis* larvae and *Nicotiana tabaccum* tobacco plant. With the amphibian model, no toxic or genotoxic effects of ashes are observed with concentrations from 0.1 to 5 g of ashes/L. If toxic and genotoxic effects of lead appear at concentration higher than 1 mg Pb/L (1 ppm), addition of only 100 mg of ashes/L neutralizes lead toxicity even with lead concentration up to 10 ppm. Chemical investigations (kinetics and X-ray diffraction (XRD) analysis) reveals that lead is quickly immobilized as pyromorphite [$Pb_{10}(PO_4)_6(OH)_2$] and lead carbonate dihydrate [$PbCO_3{\cdot}2H_2O$]. Tobacco experiments are realized on contaminated soils with 50, 100, 2000 and 10 000 ppm of lead with and without ashes amendment (35.3 g ashes/kg of soil). Tobacco measurements show that plant elongation is bigger in an ashes-amended soil contaminated with 10 000 ppm of lead than on the reference soil alone. Tobacco model points out that ashes present two beneficial actions as they do not only neutralize lead toxicity but also act as a fertilizer.

Keywords: Meat and bone meal ashes; Lead; Toxicity; Genetic effects; *Xenopus* larvae; Tobacco

1. Introduction

Bovine spongiform encephalopathy (BSE) associated with the use of meat and bone meals (MBMs) to feed cattle remains up to date one of the major public health issues that have arisen for the last twenty years [1]. Although the BSE crisis seems to end since the export of UK bovine meat has been reauthorized into the European market, MBM are still not allowed for cattle feeding. Indeed, since 1994 and according to a European decision [2], feeding ruminants with animal proteins has been banned within EU and in November 2000, this measure was extended to all cattle in France. However, MBM production is inherent to meat production and their disposal remains a major concern for the meat/food industry.

In 2004, MBM production in France reached 703 000 t [3] according to the SIFCO (French animal by-products industries union). This production is divided and classified into categories depending from their origins. Before May 2003, MBM were classified using the low- and high-risk definitions, which tended to be misunderstood by the public opinion. This classification was discarded from this date and substituted with three categories [4]. Category 1 is a high-risk material including the carcasses of animals suspected or confirmed as having BSE,

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doi:10.1016/j.jhazmat.2006.12.021

the carcasses of zoo and pet animals and the specified risk materials. Category 2 is also high-risk material, mainly carcasses of diseased animals and animals which die on farm. Category 3, former low-risk MBM, is material from safe animals, which are fit for human consumption. According to the SIFCO, more than a third of the MBM French production in 2004 was related to categories 1 and 2 (respectively 231 000 and 19 000 t) while category 3 MBM reached 453 000 t. The permitted disposal routes are nowadays defined according to these categories. Categories 1 and 2 materials are mostly incinerated. Category 3 materials are partly incinerated too. Nevertheless, their use in pet food and fertilizer industries is authorized.

Since 2000, priority is given to incineration of high-risk MBM (categories 1 and 2), mostly co-incinerated in cement kilns, which were not able to handle the whole of MBM production, including category 3. Until 2003, low-risk MBM were consequently stored for further destruction and/or valorization. From this year, the increase of the cement plants capacities across Europe and the valorization in thermal–electrical plants, especially in Germany, have allowed the destruction of the complete annual MBM production.

MBM exhibit good fuel properties with high calorific values [5], but they are mainly co-incinerated or destructed in cement kilns without any further valorization except thermal valorization. For few years, a number of studies [5–9] report the feasibility and the efficiency of MBM combustion and/or pyrolysis. In France, a call for projects relating to the valorization of MBM as combustible in specific incinerators led the state, in 2002, to select four incineration plant projects even if none has come off since. Co-combustion and incineration in cement kilns are nowadays the main disposal routes for MBM. However, co-combustion produces ash mixture that might not be a recoverable by-product as in municipal solid waste incinerators. Ashes arisen from coal and MBM co-combustion could be accepted in normal municipal landfills. However, coal addition leads to increase in heavy metal amount in the fuel [8] supplying the combustor. Thus, a specific incineration remains an alternative that could offer the opportunity to achieve both thermal valorization and solid waste recovery.

In previous papers [10–12], we investigated various valorization routes of ashes arisen from specific MBM combustion. Since these ashes are mainly calcium phosphate materials [10,11], they might be usable as agricultural soil fertilizers or as phosphate source for industry such as an additive to phosphate rocks for phosphoric acid production. MBM ashes might also be used for heavy metals uptake, which is major environmental and health concern. Many studies are aimed at lead recovery from wastewater [13–16] as well as from polluted soils [17–20]. A large number of minerals are investigated and calcium phosphate-rich materials, that is, mineral apatites, bone meals, phosphate rocks, provide effectiveness in recovering lead from contaminated soils [21–32] and wastewaters [33–41]. The resulting lead phosphates exhibit relatively low solubility [42,43] allowing immobilization of lead. The effectiveness of this immobilization must be evaluated by both chemical and biological studies. Such complementary studies enable the assessment of the bioavailability of lead meaning the rate of lead that is available for living organism [43]. Whereas chemical studies allow determining the stability, the reactivity and identifying the various lead species produced. Moreover, in vivo assays allow evaluating toxicity and/or genotoxicity effects of lead-rich materials on living animals. For years, in vivo studies are realized and there is nowadays no doubt that many sources of exposure of lead, that is, contaminated soils, paint chips, and industrial waste, are toxic to humans [44,45]. The toxic effects of pollutants have also been investigated towards amphibians [46]. In vivo studies conducted in laboratory, enable comparison of effects of various forms of lead as they allow controlling the conditions to which organisms are exposed [47]. Finally, bioavailability of heavy metals in various media can be evaluated according to their toxic and/or genotoxic effects on living organisms, both animals [48–51] and plants [52].

We report here the assessment of MBM combustion residues to immobilize lead. Ashes arisen from the combustion of MBM operated in a lab scale are characterized. The nature of the ashes produced is indeed essential to make a decision on what could be their fate. In order to evaluate the immobilization of lead by ashes and consequently the bioavailability of sequestered lead, the behavior of ashes in solution and the kinetics of lead uptake in water are preliminary studied. Toxic and genotoxic effects of lead species are afterwards evaluated by testing two different living organism models according to the medium. Amphibian larvae, *Xenopus laevis*, are chosen to evaluate bioavailability of lead in aqueous media. This model has been largely used because of its ecotoxicological relevance [53,54]. We report here chemical and new biological experiments in order to gain some insight into the mechanism, and to discuss and clarify previous published results [55]. Moreover, toxic potential of lead in soil is assessed using the model heterozygous tobacco (*Nicotiana tabaccum*, var. xanthi Dulieu). Physiological impacts such as the mortality or elongation parameters are monitored during seven weeks. Phytoavailability of lead after amendment of ashes into the soil is also investigated using the same model. Toxicity observations are clarified and compared to chemical results. At last, the toxic and genotoxic effects of MBM ashes are investigated using these two models too. The harmlessness of the ashes is indeed essential to consider any further valorization.

2. Materials and methods

MBM combustion residue: Low-risk (category 3) sterilized MBM (133 °C/3 bar for 20 min to inactivate BSE protein), were provided by Fersobio. MBM were burned twice by calcination in an electric furnace programmed to reach 850 °C at 2°/min. During combustion, MBM particles melted and sticked together. The first combustion gave a black residue (carbon-rich). This residue was mixed manually before a second combustion in order to complete decomposition and obtain clear ashes. Crude ashes were grinded, with a centrifugal mil of agate balls, and sieved at 250 μm.

Elements analysis was performed by atomic adsorption (AA) with a graphite furnace atomization (Perkin Elmer SIMA 6000) or inductively coupled plasma (ICP). Certified aqueous

standards and matrix modifier [$Mg(NO_3)_2$ and $NH_4(H_2PO_4)$] were obtained from Aldrich.

Thermogravimetric analysis was performed with a Setaram TG-DTA92, in platinum crucible, under air atmosphere, from 20 to 900 °C with an increasing temperature rate of 3 °C/min.

X-ray diffractometry was realized on powdered solids using a Siemens D501 diffractometer operating with Co Kα radiation (1.78892 nm; 30 kV; 35 mA). Measurements were made using a step-scanning technique with 2θ step intervals of 0.029° from $0.29° < 2\theta < 105°$ and an acquisition time of 1 s/step. Phases were identified by comparing the pattern with joint committee for powder diffraction standards (JCPDS) files.

FTIR spectroscopy (400–4000 cm^{-1}) of ashes was done with pellets of 1 mg of HA sample and 100 mg KBr (ATI Mattsson Genesis series FTIR).

Solid-state NMR analysis: ^{31}P cross-polarization solid-state magic angel spinning (CP MAS) spectra were recorded at 161.77 MHz, with a spinning rate of 12 kHz (Brucker avance 400 spectrometer).

Particle size analysis was performed on a laser (He–Ne at 632.8 nm) diffraction analyser (Mastersizer S – Malvern) equipped with a solution dispersion accessory (Hydro QS-MU – Malvern) in pure ethanol solution. $D(V, 0.1)$, $D(V, 0.5)$, and $D(V, 0.9)$ values are the maximum particles size for particles representing respectively 10%, 50% and 90% of sample volume.

Ionic chromatography analysis were realized on a Metrohm 761 Compact IC. Anions (SO_4^{2-}, NO_3^-, Cl^-, and PO_4^{3-}) were eluted with a mixture of $NaHCO_3$ (2.4 mmol/L) and Na_2CO_3 (2.5 mmol/L) with acetone (2 wt.%) and separated on an anionic column (Metrosep Anion Dual 1). Cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) were eluted with a mixture of dipicolinic acid (0.75 mmol/L) and tartric acid (4 mmol/L) separated on a cationic column (Metrosep C2 100).

Larvae exposure conditions: Sexually mature *X. laevis* were provided by the Developmental Biology Department of Rennes University (France). The experimental exposure conditions are described in the French Standard AFNOR NF T90-325 (AFNOR, 2000). The growth media for the amphibian is a mixture of nutritive salts (294 mg/L $CaCl_2{\cdot}2H_2O$, 123.25 mg/L $MgSO_4{\cdot}7H_2O$, 64.75 mg/L $NaHCO_3$, 5.75 mg/L KCl) in distilled water (22 ± 0.5 °C). *Xenopus larvae* were exposed in groups of 15 or 20 (100 mL/larvae) in 5 L glass flasks. They were submitted to a 12 h light, 12 h dark cycle during 12 days. They were fed every day with dehydrated aquarium fish food and the media was daily renewed. Exposure began on larvae at stage 50 of the *Xenopus* development table [56] characterized by the hind limb bud longer than broad, constricted at the base. The negative control (NC) was the growth media. The genotoxic positive control (PC) contains 20 mg cyclophosphamide/L, a well-known mutagen agent (provided by Sigma–Aldrich). PCs have been systematically performed in each experiment in order to check both the responsiveness of the amphibian larvae and that the experiment was operating correctly. For ashes, the concentrations used were 0.1, 0.5, 2.5, and 5 g/L. Lead contamination was realized with $Pb(NO_3)_2$ (99%, provided by Sigma–Aldrich) at 0.001, 0.01, 0.1, 1, 10, 30, 50, and 100 mg Pb/L. For immobilized lead on ashes toxicity evaluation, ashes concentrations were 0.1 g/L.

Micronucleus test, *genotoxicity assay*: A blood sample was obtained from each anaesthetized larva by cardiac puncture with heparinized micropipettes (20% solution at 5000 IU/mL) at the end of the 12 days of exposure. After fixing in methanol and staining with hematoxylin, the smears were screened under the microscope (oil immersion lens, ×1500). The number of erythrocytes that contained one micronucleus or more was determined in a total sample of 1000 erythrocytes per larva. Slides were blind scored by only one individual. 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. The medians and quartiles were then calculated. The statistical method used to compare the medians was based on the recommendations of McGill et al. [57] and consists in determining the theoretical medians of samples of size n (where $n \geq 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times IQR/\sqrt{n}$, where M is the median and IQR is the inter-quartile range (upper quartile − lower quartile). Under these conditions, the difference between the theoretical medians of the test groups and the theoretical median of the NC group is significant to within 95% certainty if there is no overlap.

Tobacco experiment: Standard soil ISO composition is nearly 70% of quartz sand (with particle size mainly lower than 200 μm), 20% of kaolin clay (with kaolinite content >30%), 10% dried matter of acid (sphaigne tourb) and 0.6% of calcium carbonate. Residual humidity level is 34.4% and pH 6.25. Ten conditions were studied and 3.4 kg of soil were used for each condition. For ashes-amended soil experiments 120 g of ashes were mixed with 3.4 kg of soil. Then, all soils were flooded with distilled water (1 L/kg of soil) containing different lead concentrations (0, 50, 100, 2000, or 10 000 ppm). They were incubated for 20 days at room temperature and manually homogenized each day. Drying was achieved by eating 4 h at 60 °C. Before use, soils were sieved at 1 mm. Tobacco plants (*N. tabaccum*, var. xanthi Dulieu) were produced in vitro in the laboratory using culture medium 169 (Murashige and Skoog solutions supplemented with EDTA-Fe and biotin at pH 5.5). At the 6–8 leaf stage, each plant was placed in a pot filled with 170 g of soil. Twenty plants were cultivated for each condition. All conditions were then maintained in a greenhouse receiving filtered air (over a synthetic filter and activated charcoal) under constant overpressure, at 20–25 °C with a 12 h per day photoperiod. Each pot received 40 mL of water a day. Each week, physiological impacts such as the mortality or elongation parameters (height of plant pressure) were monitored.

3. Preliminary chemical investigation – results and discussion

3.1. Ashes characterization

Ashes used in this work are produced by calcination at 850 °C of low fat MBM coming from slaughterhouse waste. According to European standards, MBM were previously dehydrated

Table 1
MBM combustion residue composition

Ashes composition	
Main compounds content (wt.%)	
Water[a]	0.30
Phosphates[b]	57.5
Main elements content (wt.% > 1%)	
Ca[c,d]	31.00
P[c]	18.77
Na[c]	2.43
K[c]	1.49
Cl[c]	2.01
Traces elements content (wt.% < 1%)	
Mg[c]	0.67
Zn[c]	0.04
Cu[c]	0.01
Si[c]	0.69

[a] ATG measurement.
[b] Calculated value (considering all phosphorus are coming from phosphates).
[c] ICP measurement.
[d] AA measurement.

(110 °C/4–5 h) and sterilized (133 °C/20 min/3 bar). The resulting low-risk MBM still contains water (3–8 wt.%) but also large amounts of fats (10–14 wt.%) and others organic compounds (25–35 wt.%) allowing their combustion [3].

Chemical analysis shows that ashes are calcium (31.0 wt.%) and phosphate (57.5 wt.%) rich material (Table 1). Significant levels sodium, potassium, chloride, magnesium, and silicon are also observed. These results are in accordance with published results [6,11].

Comparison of X-ray diffraction (XRD) pattern to JCPDS file shows the presence of a major calcium hydroxyapatite (CaHAP) phase [$Ca_{10}(PO_4)_6(OH)_2$] (Fig. 1). Contrary to a previous work, with ashes produced by incineration at a lower temperature (550 °C), tricalcium phosphate phase (β-TCP) is not observed [11]. In IR spectrum, mainly strong split phosphate (PO_4^{3-}) bands are observed in the range 1100–1000 cm^{-1} (stretching mode) and 500–600 cm^{-1}. These bands are characteristics of mineral phases of calcified tissues like bone or teeth, CaHAP [$Ca_{10}(PO_4)_6(OH)_2$] being their major inorganic constituent [58]. Moreover, a small band at 879 cm^{-1} attributed to (HPO_4^{2-}) indicates the presence of calcium deficient apatite: $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$. These results are in agreement with ^{31}P {1H} MAS NMR spectrum (Fig. 2) as a strong signal at 3.3 ppm (line with 110 Hz) characteristic

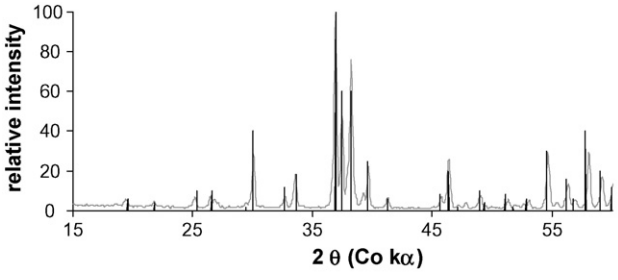

Fig. 1. XRD pattern of MBM ashes (vertical lines represent JCPDS pattern of HAP).

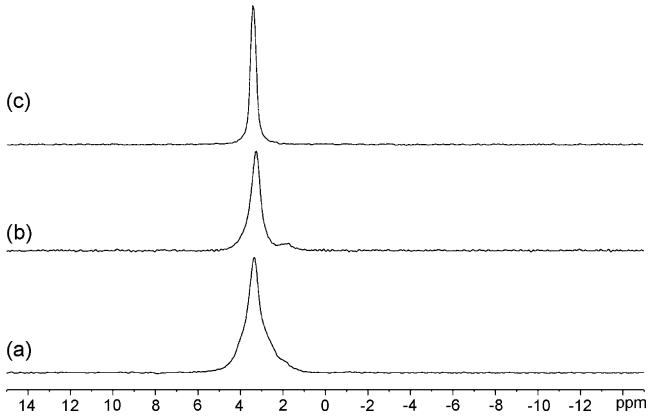

Fig. 2. (a) ^{31}P MAS NMR spectra. of ashes. (b) Cross-polarization ^{1}H-^{31}P MAS NMR spectra of ashes. (c) ^{31}P MAS NMR spectra of HAP.

of CaHAP is observed. The position and the shape of the signal do not fit with those of crystalline α-tricalcium phosphate, β-tricalcium phosphate, or tetracalcium phosphate [58–60]. Cross-polarization $^{1}H-^{31}P$ MAS analysis give a better resolution (line with 85 Hz) and a much smaller signal is observed around 1.8 ppm. This small signal could be attributed to HPO_4^{2-} ions, confirming the presence of calcium deficient apatite [$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$] as observed in IR analysis.

Particle size distribution of ashes is relatively broad, ranging from a few micrometers to a few milimeters, with irregular shape. After sieving, we observe that 11 wt.% of ashes particles are bigger than 1 mm, 32% between 250 μm and 1 mm, and the remaining 57% smaller than 250 μm. Crude ashes were grinded and sieved at 300 μm before use to get a homogenous material. Particle size analysis shows a large dispersion with $D(V, 0.1) = 8.2$ μm, $D(V, 0.5) = 105.9$ μm, and $D(V, 0.9) = 269.2$ μm. Moreover, sieving gives particles small enough to allow their ingestion by larvae and to get so an acute evaluation of ashes toxicity.

3.2. MBM ashes solubility

Previous to biological studies we investigate first MBM ashes behavior in water. With amphibian model, the growth media is indeed rich in ionic species and solubility can be strongly modified compared to the one in deionized water. As expected from elemental and structural analysis, ashes are poorly soluble in *Xenopus* growth media. We observe no more than 9% dissolution after 24 h (solid/liquid ratio in weigh equal to 1000). Ionic chromatography monitoring of ashes dissolution in distilled water, indicates that released ions are mainly Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}, Cl^{-}, PO_4^{3-}, or SO_4^{2-}, coming from very soluble salts such as NaCl, KCl, ... as observed in previous work [11]. The remaining solid is the HAP phase which solubility is very low at the experimental pH [58,61]. These firsts results indicate that even at concentration up to 5 g ashes/L, water quality remains excellent for larvae growth, and pH (7.2–8.3), Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}, Cl^{-}, PO_4^{3-}, and SO_4^{2-} concentrations remain, according to European standard, within limits for water to human consumption.

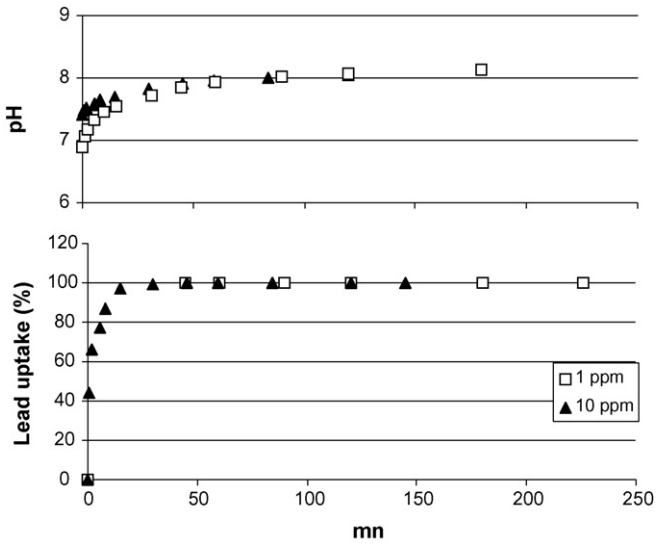

Fig. 3. pH and lead uptake (wt.%) by MBM ashes (0.1 g/L) for Pb^{2+} initial concentration of 1 and 10 ppm.

3.3. Kinetic of lead removal by MBM ashes

In a previous paper, we showed that removal of dissolved Pb ions by ashes (in deionized water) is rapid [10]. The mechanism involves at least three successive steps: surface complexation of lead, $Ca_{10}(PO_4)_6(OH)_2$ dissolution followed by precipitation of $Pb_{10}(PO_4)_6(OH)_2$ and slow Pb diffusion/substitution of Ca. The first two steps occur rapidly and a high lead uptake capacity of 275 mg/g ashes is reached in less than 3 h (in deionized water), whereas reaching the total lead immobilization capacity would require more than 10 days [10]. Such mechanism is similar to the one involved in lead immobilization on hydroxyapatite [39] and can be assimilated to a substitution of calcium by lead. In the present study, the aqueous media in which larvae are exposed contains high level of ions such as calcium with a molar concentration 13 times higher than the highest lead concentration used for genotoxic evaluation (30 ppm). This media should modify both kinetic and lead immobilization mechanism.

In order to evaluate the incubation time, we first realized a kinetic study of lead removal by ashes in the *Xenopus* larvae growth media. Fig. 3 presents lead uptake by ashes and pH evolution versus time for two solutions at 1 and 10 ppm initial lead concentrations. These results show that for both concentrations almost 99% of lead is immobilized within 30 min and the residual lead concentration after 20 h is lower than 0.01 ppm. A similar experiment is realized with a 30 ppm lead solution, which corresponds to a slightly higher quantity than ashes capacity. But in this case too, the residual Pb^{2+} concentration after 20 h remains very low (0.13 ppm). For biological test, ashes are consequently incubated during 20 h in lead solutions before adding *Xenopus* larvae. At this point, it is interesting to notice that, with solution at 1 and 10 ppm, the residual Pb^{2+} concentration remains lower than European standards for human consumption water, underlying ashes efficiency to extract lead from an aqueous solution.

4. Toxicity and genotoxicity investigation. Results and discussion

4.1. Evaluation of MBM ashes toxicity and genotoxicity on Xenopus larvae

Amphibian larvae have been exposed to various concentrations of ashes (0.1; 0.5; 2.5 and 5 g/L) for 12 days. Severe toxicity (death) and weak toxicity (abnormal behavior, reduced size, diminished food intake) were evaluated by visual inspection. The first observation points out that ashes are not toxic at all to amphibian larvae in the explored experimental conditions. They even seem to boost larvae growth, by comparison with the NC. Moreover, dissection of larvae, after 12 days exposition, shows the presence of ashes in their gut, confirming their ingestion by *Xenopus*. Even if ashes particles are not very soluble in operating conditions, the low pH in gut should increased their dissolution [61] and consequently possible release of toxic substances as well as nutrition supplement such as calcium, sodium, phosphates.

At last, micronucleus test results (Fig. 4) do not reveal any genotoxicity of ashes on larvae. Indeed, for each group of animals, the number of MNE ‰, which is the result of genetic toxicity, is lower than NC group. This experiment demonstrates MBM ashes harmlessness towards larvae (at concentrations ranging from 0.1 to 5 g/L). These results tend to show that MBM ashes spread could actually be achieved with the aim of environmental applications without any negative impact.

4.2. Evaluation of lead toxicity and genotoxicity on Xenopus larvae

Amphibian larvae have been exposed for 12 days to eight leads concentrations: 0.001, 0.01, 0.1, 1, 10, 30, 50, and 100 mg Pb/L (ppm). Severe toxicity appears at 10 mg/L and lethality rate increases afterwards with lead concentration (Fig. 5). Moreover, amongst survival larvae, arise physical signs of anaemia, lower size, restricted food intake or abnormal behavior (perturbed swimming). Weak toxicity is observed at 1 mg/L (anaemia, diminished diet, and size). With lower concentrations no toxicity is observed.

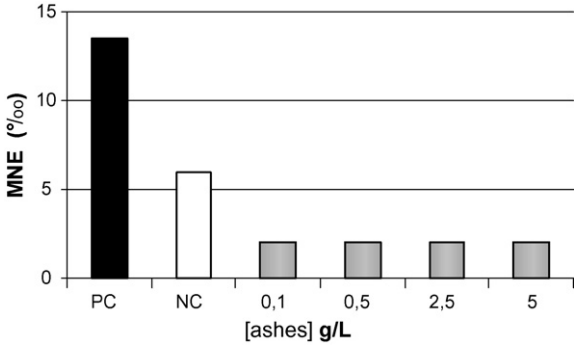

Fig. 4. Micronucleated erythrocytes per thousand values (MNE ‰) after incubation of *Xenopus* larvae in different conditions: positive control (PC), negative control (NC) and with ashes at various concentrations (0.1, 0.5, .2.5, and 5 g ashes/L).

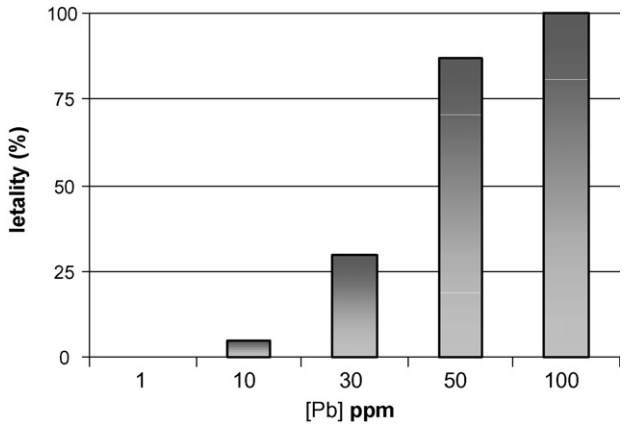

Fig. 5. *Xenopus* larvae letality (%) after incubation in various lead concentration solution.

For genotoxic assessment, lead concentrations are limited to 0.01, 0.1, 1, and 10 ppm because of the acute toxicity observed at higher doses. The NC leads to an MNE ‰ value equal to 6‰. Significant higher MNE, 14 and 202‰, are observed for 1 and 10 mg Pb/L, respectively. The lowest Pb concentrations are not genotoxic towards *Xenopus* larvae.

Under those experimental conditions and according to the evaluated concentration range, toxic, and genotoxic effects on larvae are observed with lead concentrations higher than 0.1 ppm.

4.3. Evaluation of toxicity and genotoxicity of immobilized lead on MBM ashes on Xenopus larvae

The above data reveal ashes harmlessness towards *Xenopus* larvae, up to 5 g/L. However, evaluation of the toxicity of lead after immobilization on ashes matrix is necessary and essential to know whether ashes can be safely used to remove lead from wastewater. Comparison with the previous results on lead nitrate toxicity should give information on bioavailability of this sequestered lead.

Amphibian larvae were exposed during 12 days to lead (six lead concentrations: 0.001, 0.01, 0.1, 1, 10, and 30 ppm) after a 20-h contact time of the initial lead solutions with ashes (100 mg/L). Observation of acute toxicity of larvae does not show any sign of severe, neither weak, toxicity. Moreover, in comparison with NC group, and in opposition with what occurs for some equivalent lead concentrations without ashes, no difference, such as abnormal behavior, reduced size, or diminished food intake, is observed on larvae. This first result demonstrates the high efficiency of ashes to inhibit lead toxicity towards larvae.

Genotoxicity assessments of lead solutions, with and without ashes addition, are presented on Fig. 6. If lead genotoxicity appears at 1 ppm (MNE = 14‰) and increases dramatically at 10 ppm (MNE = 202‰), addition of only 0.1 g of ashes/L allows to neutralize lead genotoxic effects, even at concentration up to 10 ppm Pb (MNE = 3‰). Moreover, with ashes, genotoxicity appears only from 30 ppm of lead (MNE = 24‰) which underlines their efficiency to immobilize Pb^{2+} ions and to

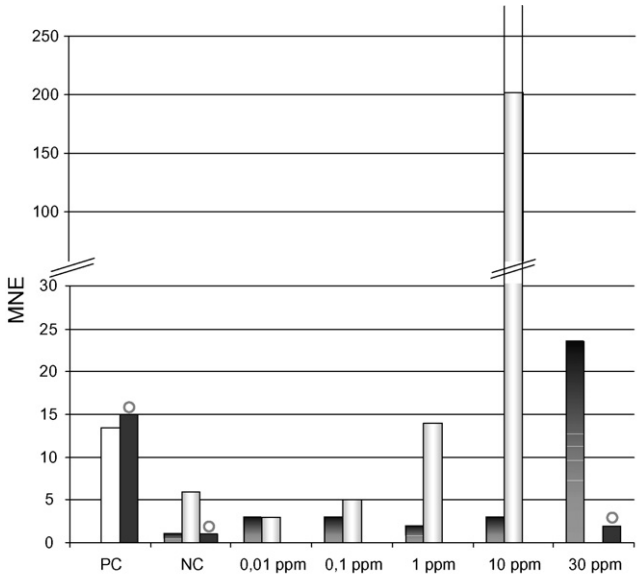

Fig. 6. Micronucleated erythrocytes per thousand (MNE ‰) values after incubation of Xenopus larvae in different condition: positive control (PC), negative control (NC), and with various lead concentrations (0.01, 0.1, .1, 10, and 30 ppm). Grey rectangle represents experiments without addition of ashes and black rectangle with ashes. Grey circles indicate experiments realized with ashes previously incubated with lead in acidic pH.

decrease their bioavailability. One can also notice that the number of MNE ‰ of larvae incubated with 30 ppm Pb and ashes remains very low compared to the one at 10 ppm without ashes (MNE = 202‰). It is actually close to MNE ‰ of larvae incubated with 1 ppm of lead. However, in those conditions one could expect a much lower MNE value. Indeed, kinetic study indicates that the remaining lead concentration, when ashes are incubated in 30 ppm Pb^{2+} growth media solution, is 0.13 ppm what should then induce a MNE around 5‰ instead of 24‰. According the hypothesis that the lead final concentration should not be modified when tests with larvae are conducted, the observed value indicates that genotoxicity could be induced by another species than ionic lead.

From the above kinetic study, one can discuss the mechanism of lead immobilization by ashes in the growth media to explain these biological results. The reaction of calcium phosphates with dissolved lead ions is described as rapid with a mechanism involving apatite dissolution followed by lead insoluble salts precipitation [39]. Published results have shown that the dissolution of apatite is correlated to precipitation of pyromorphite $[Pb_{10}(PO_4)_6(X)_2]$ (X = Cl, OH, F depending of ions in solution) under rather acidic condition, while lead carbonates, such as hydrocerussite $[Pb_3(CO_3)_2(OH)_2]$ are favored under alkaline conditions [61,62]. In agreement with literature results, we demonstrate, in a previous work [10], that lead ions immobilization by ashes, involves precipitation of hydroxypyromorphite $[Pb_{10}(PO_4)_6(OH)_2]$. Nevertheless, these experiments were realized in demineralized water under slightly acidic condition ($4.4 < pH < 6.9$), whereas here, experiments are realized in an ions-rich solution under slightly alkaline conditions ($7.2 < pH < 8.3$). In order to elucidate the mechanism involved in

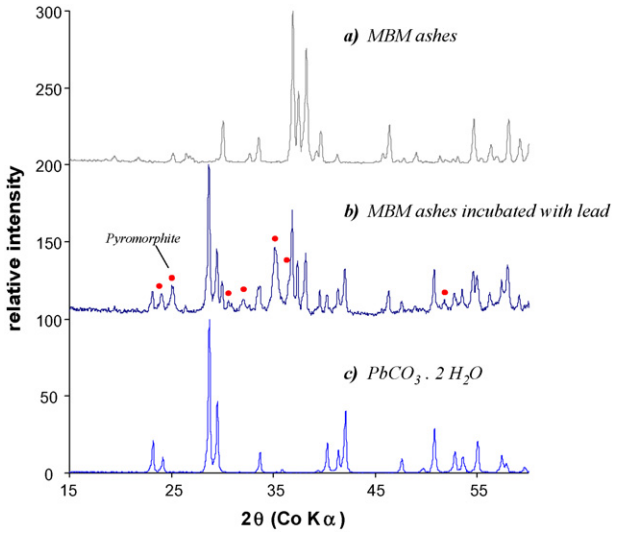

Fig. 7. XRD pattern of (a) MBM ashes, (b) ashes incubated with lead, and (c) lead carbonate.

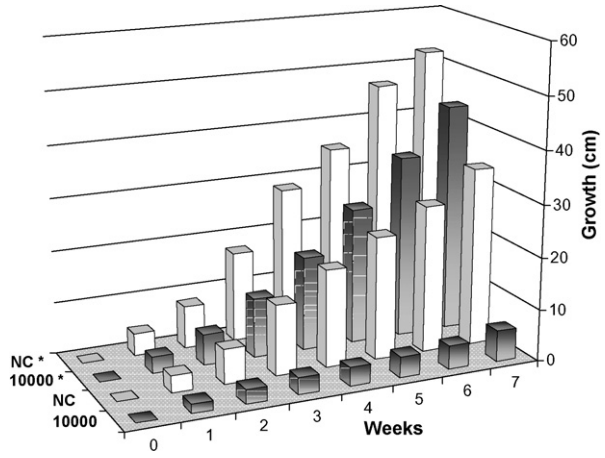

Fig. 8. Tobacco growth of negatives controls (NC) and lead-contaminated soils at 10 000 ppm (10 000) with (*) and without ashes amendment.

the toxicity test conditions, we realized an XRD analysis of the residual solids 20 h after incubation of 500 mg of ashes in 30 ppm lead solution (5 L). Comparison of XRD pattern of the residual solid (610 mg) to JCPDS files shows the presence of three major phases: hydroxyapatite, pyromorphite [$Pb_{10}(PO_4)_6(OH)_2$], and lead carbonate dihydrate, [$PbCO_3{\cdot}2H_2O$] (Fig. 7). If pyromorphite formation is the result of lead immobilization by CaHAP, lead carbonate crystallization highlights another mechanism probably induced by the growth media composition. And indeed, when a growth media solution containing 30 ppm of Pb^{2+} ions (without ashes) is prepared, it becomes slightly cloudy during lead addition and a white powder can be collected by filtration (final pH 6.3). Comparison of XRD pattern of the white precipitate to JCPDS files shows the presence of one single phase: lead carbonate [$PbCO_3$, $2H_2O$]. Therefore, could the difference between the presumed genotoxicity at 30 ppm with ashes and the measured one arise from the formation of lead carbonate? Precipitation of $PbCO_3$, $2H_2O$ allows decreasing of not only ionic lead concentration, which is benefit, but also pyromorphite phase amount. Thermogravimetric analysis (TGA) of the solid phase, by measurement of mass loss induced by lead carbonate dehydratation (around 270 °C), indicates that $PbCO_3{\cdot}2H_2O$ represents nearly 13.7% in weight. Moreover, dissection of larvae, after 12 days of exposition, confirms the presence of swallowed solid particles in their gut where the low pH might increase solid particles dissolution. As solubility of lead carbonate is higher than pyromorphite, lead would be released and consequently bioavailable. From these results, one can assume that lead in pyromorphite is less bioavailable than in lead carbonate. To confirm this hypothesis, ashes were incubated with lead in acidic condition (pH < 6.5) and DRX analysis of the resulting powder confirms that only CaHAP and pyromorphite are obtained. *Xenopus* larvae were then incubated for 12 days with this lead loaded precipitate (2 g/L in order to have a lead contain of 30 ppm). Micronucleus test results (Fig. 6) show a strong decrease of the number of MNE (2‰) by comparison to the former experiment (MNE = 24‰). This lower MNE value is closer to the expected value (MNE = 3‰ for 0.1 ppm lead solution without ashes) considering that genotoxicity is induced by the remaining soluble Pb^{2+} ions (0.13 ppm).

These results on toxicity and genotoxicity of lead towards *Xenopus* larvae demonstrate the ability of MBM ashes to extract lead and consequently to decrease its bioavailability in aqueous media.

4.4. Preliminary evaluation of MBM ashes to immobilized lead in soils

According to ashes efficiency to immobilize Pb^{2+} ions in solution, their use for lead-contaminated soil remediation could be a promising valorization way to explore. In the present study, we examine the effect of ashes amendment (35.3 g of ashes/kg of soil) on tobacco culture in lead-contaminated soils.

Fig. 8 represents tobacco elongation on four different soils: reference soil alone (NC), soil amended with ashes (NC*), soil containing 10 000 ppm of lead without ashes amendment (10 000), and with ashes (10 000*). Soils amended by ashes are identified with a star. First of all, these experiments highlight and confirm the very high toxic effects of lead-contaminated soil (10 000 ppm) on tobacco plants since the growth is very limited. Moreover, comparison of NCs (NC and NC*) demonstrates the beneficial action of ashes, as tobacco elongation in amended soil is faster and longer than in soil alone. This could be explained by soluble phosphate dissolution, a major component of ashes as well as of fertilizers. Last but not least, the comparison of 10 000* with NC underlines the great efficiency of MBM ashes to immobilize lead in contaminated soil as tobacco elongation is even faster than in the non-amended reference soil. This last result points out that ashes amendment enables two beneficial actions on tobacco elongation since it does not only neutralize lead toxicity but also does fertilize the soil.

With lead-contaminated soil at lower concentration (50, 100, and 2000 ppm) tobacco elongation is faster and longer (30–60% longer) in ashes-amended soil (Fig. 9). But surprisingly lead concentrations up to 2000 ppm, in non-amended soil, seem not to modify dramaticaly tobacco plants growth as their elongations

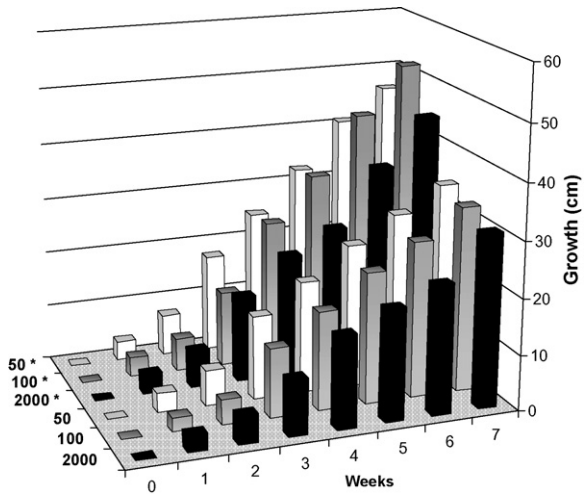

Fig. 9. Tobacco growth on lead-contaminated soil at 50, 100, and 2000 ppm with (*) and without ashes amendment.

are similar to, or slightly smaller than, in the reference soil (NC) whatever the lead concentration amongst the three ones. This last observation leads us to investigate the reason why tobacco test seems not to be very sensitive to lead contamination up to 2000 ppm. Three hypothesis can be suggested:

- tobacco plants develop biological mechanisms such as defense against lead absorption;
- most of the lead is lixiviated from pots by irrigation during the experiments;
- lead is partially immobilized by the soil itself.

To confirm or deny the two last ones, a kinetic study on lead immobilization by soil is realized. Thus, 2.5 g of soil are mixed within 500 mL of Pb^{2+} solution (in deionized water). Fig. 10 presents lead uptake (wt.%) by soil and pH evolution versus time for initial solutions at 25, 50, 75 and 100 ppm of Pb^{2+}. With

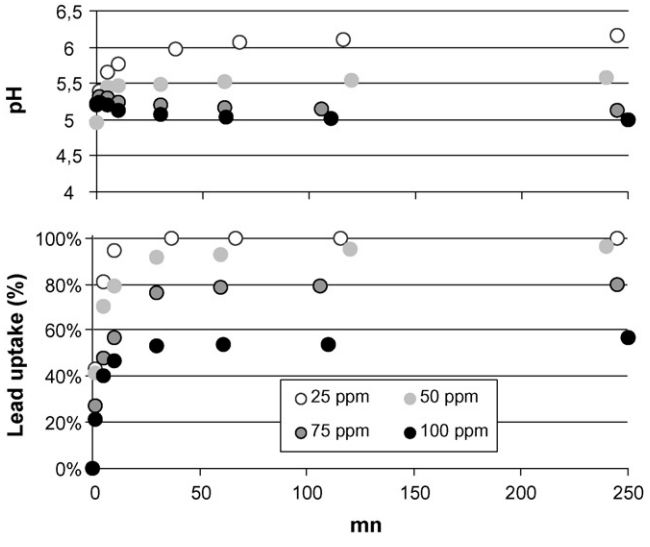

Fig. 10. pH and lead uptake by soil for Pb^{2+} initial concentration of 25, 50, 75, and 100 ppm.

25 ppm solution, 100% of lead is immobilized within 60 mn and the residual lead concentration is lower than 0.1 ppm. Moreover, after 0.2 μm filtration, this soil was added in 500 mL of deionized water and even with a liquid to solid ratio equal to 200, no lead release was observed. These results underline, on the one hand, the strong ability of soil to remove Pb^{2+} ions from acidic solutions and, on the other hand suggest that no lixiviation, due to irrigation, occurs. At last, experiments with higher lead concentration (Fig. 10) allow us to evaluate a maximum capacity of nearly 11 mg of Pb/g of soil. Considering those results, the strong toxicity observed only with contaminated soil at 10 000 ppm (10 mg Pb/g of soil), which is close to maximum lead immobilization capacity by soil, could be explained by the presence of residual soluble or free lead in soil. With lower lead concentrations, most of the metal would be immobilized by soil neutralizing so its toxicity. However, biological defense mechanisms could also be involved.

The difference of elongation observed on tobacco between lead-contaminated soils (50, 100, and 2000 ppm), with and without ashes amendment is more difficult to explain as both soil and ashes have good lead immobilization capacities. On the one hand, lead could be more bioavailable in crude soil than when ashes are amended. On the other hand, bioavailability of lead could be rather the same and the difference would only arise from the fertilizing properties of ashes. At this point, no answer can be given. Bioaccumulation measurements will be carried out to allow us to conclude. However, even if soil composition appears not to be ideal for lead immobilization studies as it competes with ashes, this work underlines the efficiency of MBM ashes to immobilize lead in contaminated soil.

5. Conclusion

The above results demonstrate MBM combustion residue harmlessness towards the studied biological models: amphibian *X. laevis* and *N. tabaccum* tobacco plant. In both case, they even seem to boost their growth.

Toxicological studies on *Xenopus* larvae underline ashes efficiency to immobilize lead in solution and decrease its bioavailability. Indeed, if a 1 ppm lead solution is genotoxic, a 10 ppm lead solution containing 0.1 g of ashes/L is harmless. Mechanistic study reveals that, in our experimental conditions, lead is immobilized as pyromorphite [$Pb_{10}(PO_4)_6(OH)_2$] and lead carbonate dihydrate [$PbCO_3{\cdot}2H_2O$]. Genotoxic experiments indicate that lead is more efficiently immobilized in pyromorphite phase than in the higher soluble lead carbonate phase. Additional study should be carried out to determine lead biodisponibility of these two compounds.

Similar efficiency is observed on tobacco model. The results point out that ashes present two beneficial actions as they do not only neutralize lead toxicity but also act as a fertilizer. Indeed, plant elongation in ashes-amended soil is unaffected by lead contamination up to 10 000 ppm. However, a kinetic study shows that the soil itself reveals some lead immobilization ability that hides ashes efficiency at lower concentration. Additional studies on bioaccumulation are developed in order to evaluate the bioavailability of lead immobilized on ashes.

This work tends to demonstrate that MBM combustion residue could be used for environmental application such as in situ lead immobilization in water, contaminated soil, or agricultural soil enrichment.

Acknowledgements

The authors wish to thank Fersobio SA for providing MBM used in this work. We would like also to thank the Chemistry Department of "IUT Paul Sabatier" for financial support and for offering accesses to some analytical equipment, L. Orts and T. Scandella for laboratory assistance.

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