Journal of Hazardous Materials 166 (2009) 945–953

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Evaluation of laboratory and industrial meat and bone meal combustion residue as cadmium immobilizing material for remediation of polluted aqueous solutions: *"Chemical and ecotoxicological studies"*

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ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 4 November 2008
Accepted 27 November 2008
Available online 3 December 2008

Keywords:
Meat and bone meal
Incineration
Cadmium
Toxicity
Xenopus larvae

ABSTRACT

Meat and Bone Meals (MBM) combustion residues (ashes) are calcium and phosphate-rich materials. The aim of this work is to evaluate ashes efficiency for remediation of cadmium-contaminated aqueous solutions, and to assess the bioavailability of cadmium on *Xenopus laevis* larvae. In this study both industrial (MBM-BA) and laboratory (MBM-LA) ashes are compared regarding their efficiency. Kinetic investigations reveal that cadmium ions are quickly immobilized, with a maximum cadmium uptake at 57 mg Cd^{2+}/g of ashes for MBM-LA, two times higher than metal uptake quantity of MBM-BA, in our experimental conditions. Chemical and X-ray diffraction analysis (XRD) reveal that Cd^{2+} is mainly immobilized as $Ca_{10-x}Cd_x(PO_4)_6(OH)_2$ by both ashes, whereas otavite, $Cd(CO_3)$, is also involved for MBM-LA in cadmium uptake. Otavite formation could be explained by the presence of carbonates in MBM-LA, as observed by IR. Genotoxicity of cadmium solution on *Xenopus* larvae is observed at 0.02, 0.2 and 2 mg Cd^{2+}/L. However addition of only 0.1 g/L MBM-LA inhibits these effects for the above concentration values whereas Cd^{2+} bioaccumulation in larvae's liver is similar for both experiments, with and without ashes.

1. Introduction

Bovine spongiform encephalopathy (BSE) associated with the use of Meat and Bone Meals (MBM) to feed cattle remains one of the major public health issues that have arisen for the last 20 years [1]. As a result of BSE crisis, MBM valorization has been widely investigated. Among the different processes studied, specific incineration or co-incineration [2–6] remains an alternative that could offer the opportunity to achieve both thermal valorization and solid waste recovery.

In previous papers [7–12], we investigated various ways of valorization for MBM ashes. Since specific MBM combustion ashes are mainly calcium phosphate materials [7–10], essentially calcium hydroxylapatite (CaHAP), $Ca_{10}(PO_4)_6(OH)_2$, and whitlockite, $Ca_3(PO_4)_2$, they might be used 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. This previous work demonstrated their efficiency for *in-situ* remediation of lead-contaminated aqueous solution and soil using chemical and biological assay [10,11]. Toxicological studies on *Xenopus* larvae and kinetic experiments showed that ashes immobilize lead in solution and decrease its toxicity. Indeed, if a 1 mg/L lead solution is genotoxic, a 10 mg/L lead solution containing only 0.1 g of ashes/L is harmless. Study of mechanisms reveals that, in those experimental conditions, lead is immobilized as hydroxypyromorphite $[Pb_{10}(PO_4)_6(OH)_2]$ and lead carbonate dihydrate $[PbCO_3, 2H_2O]$. Genotoxic experiments indicate that lead is more efficiently immobilized in hydroxypyromorphite phase than in the more soluble lead carbonate phase. More recently, we focused on industrials MBM ashes, produced by co-incineration with other wastes [12,13]. As expected, they mainly contain CaHAP and whitlockite, but addition of other wastes (5–10% weight) during incineration increases heavy metal contamination of ashes. However, chemical analysis and leaching experiment

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doi:10.1016/j.jhazmat.2008.11.104

results showed that these industrial MBM ashes may be considered as non-hazardous material, according to European classification waste to be landfilled [14,15]. These results tend to demonstrate that industrial MBM ashes may be used for environmental applications such as *in situ* heavy metal immobilization.

According to World Health Organisation, cadmium is one of the 10 metals of most immediate concern. It is a toxic element which is recognized as carcinogen for humans [16,17]. Many studies are aimed at cadmium recovery from wastewater [18] as well as from polluted soils [19]. A large number of organic [20–24] and mineral [25] materials are investigated and calcium phosphate-rich materials, i.e. bone char [26–28], bones [29], phosphate rocks [30–31] and particularly mineral apatites like CaHAP [32–50] provide effectiveness in recovering cadmium from contaminated soils [48–50] and wastewaters [32–47]. According to literature, the immobilization of Cd^{2+} ions with CaHAP could be explained by three mechanisms, similar to those involved in lead removal by CaHAP: surface adsorption of Cd^{2+}, CaHAP dissolution followed by CdHAP precipitation or ion exchange. For some authors, Cd^{2+} sorption by HAP involves all three mechanisms [28,30–35,38–44,46–48].

Moreover, the effectiveness of Cd^{2+} immobilization must be evaluated by both chemical and biological studies that enable the assessment of the bioavailability of cadmium, meaning the rate at which cadmium is available to living organisms. Chemical studies allow determining the stability, the reactivity and identifying the various cadmium species produced, whereas, *in vivo* assays allow evaluating toxicity and/or genotoxicity effects of cadmium-rich materials on living animals.

We report here the assessment of two MBM combustion residues to immobilize cadmium. Industrial ashes (MBM-BA) are bottom ash produced by co-incineration of 95% (by weight) of MBM, coming from pork production, with 5% of additional wastes, such as plastic bag or sewage sludge, in an incineration plant. Laboratory ashes (MBM-LA) arise from the combustion of MBM coming from slaughterhouse waste, operated in an electric furnace. In order to evaluate the immobilization of cadmium by ashes and subsequently the biological effect on *Xenopus* larvae of sequestered Cd^{2+}, the kinetics of cadmium uptake and then its release in water are studied first. X-ray diffraction (XRD) experiments combined with Rietveld analysis of XRD patterns and Langmuir isotherm modeling are realized to characterize more accurately the cadmium uptake mechanism by MBM combustion residues in water. The results are compared to published results on Cd^{2+} interaction with CaHAP. Finally, acute toxicity (mortality, growth) and genotoxicity (induction of micronucleus) of cadmium are evaluated using an amphibian model, *Xenopus laevis*. This model has been largely used because of its ecotoxicological relevance [50–58].

2. Materials and methods

2.1. Laboratory MBM combustion residue (MBM-LA)

Low risk (category 3, according to [14,15]) sterilized MBM (133 °C/3 bars for 20 min to inactivate BSE's protein according to European standards), coming from slaughterhouse waste, were provided by Fersobio (France). MBM were burned twice by calcination in an electric furnace under air atmosphere: an initial combustion of 3 h at 600 °C to eliminate the organic fraction of the material, and a second combustion from 25 to 850 °C at a rate of 2 °C/min. During combustion, MBM particles melted and stuck together, inducing pyrolysis of organic matter trapped inside. 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. The amount of residue represents nearly 24% weight of initial MBM. Crude ashes were grinded to 300 μm and their specific surface BET was 4 m^2/g.

2.2. Industrial MBM combustion residue (MBM-BA)

The industrial ashes were bottom ash (MBM-BA) coming from an incineration plant equipped with a rotary furnace (12 m long). This incinerator had a capacity of 2 tons/h and operates at 1000 °C. The incinerator was fed with 95% of MBM from pork production (category 3), the remaining 5% being composed of additional wastes such as plastic bags or sewage sludge. The amount of residue represents between 10% and 15% per mass of initial product. Two batches of ashes were used and mixed together to minimize the variability of industrial production. Crude ashes were grinded to 300 μm and their specific surface BET was 1 m^2/g.

Elemental analysis was performed by atomic adsorption (AA) with a graphite furnace atomisation (PerkinElmer SIMA 6000). Certified aqueous standards and matrix modifier [$Mg(NO_3)_2$ and $NH_4(H_2PO_4)$] were obtained from Aldrich. Analysis were performed using PerkinElmer recommended conditions (pretreatment temperature: 700 °C; atomisation temperature: 1400 °C; matrix modifier addition in samples: 0.05 mg $NH_4(H_2PO_4)$ + 0.003 mg $Mg(NO_3)_2$). Calibration was realised with 2, 4, 6, 8 and 10 ppb standard solution and reproduced every 15 samples and at the end of the run. Each sample was analysed three times. The detection limit was 0.1 μg Cd^{2+} L^{-1}.

Specific surface area measurements were realised applying one-point BET method (nitrogen adsorption) on Micrometrics Model 2100E Accusorb.

X-ray diffractometry was realized on powdered solids using a Siemens D5000 diffractometer equipped with a rear monochromator and using Kα (λ = 1.789 Å) cobalt radiation. Measurements were made with a 2θ step intervals of 0.02° (5°–70°) and an acquisition time of 10 s per step. Phases were identified by comparing the pattern with JCPDS files (Joint committee for powder diffraction standards).

Rietveld method was used to quantify mineralogical compositions of materials with the developed JAVA-based Rietveld software MAUD [59]. The Rietveld refinement is an analytical method in X-rays powder diffractometry based on a least squares approach to refine a calculated line profile in comparison with XRD diffraction pattern measured [60].

2.3. Chemical investigations

2.3.1. Kinetic experiment

Kinetics were conducted in a 250 mL-reactor, at 20 °C, by mixing 200 mL of metal solution at various concentrations (25, 50, 100, 300, 1000 and 1500 mg Cd/L) with 500 mg of ashes under vigorous agitation. pH was monitored. Aliquots of the supernatant solution (one for each time) were taken using a 2 mL propylene syringe equipped with a 0.45 μm filter. Those experiments were reproduced twice. The collected samples were acidified with nitric acid and diluted before analysis at atomic adsorption. The amount of sorbed metal per gram of ashes (q_t) at time t was calculated as follows: $q_t = (C_0 - C_t)/m_{ads}$. C_0 and C_t were the metal concentration in liquid phase at the start and time t (in mg/L or mmol/L), and m_{ads} was the sorbent amount in solution (g/L).

2.3.2. Leaching experiment

After mixing ashes with Cd^{2+} solution for 24 h to make Cd-saturated ashes, the solid residue was filtered, dried (40 °C) and added to stirred distilled water solutions (solid/liquid ratio of 1/400). After 24 h, the solution was recovered by filtration

Table 1
Main elements composition (wt.%) of MBM-BA and MBM-LA (LOI: loss on ignition; tr: trace).

	Ca	P	Si	Al	Mg	Fe	Na	K	SO_4^{2-}	Cl^-	LOI
MBM-BA	28.8	16.2	3.7	1.1	1.2	1.5	2.7	1.7	0.8	0.3	0.3
MBM-LA	28.2	18.9	0.7	tr	0.7	0.1	2.4	1.5	0.4	2.0	3.6

(0.45 μm), acidified with nitric acid and diluted before cadmium titration by atomic adsorption (g/L).

2.4. Ecotoxicological investigations

In amphibian larvae, as in most eukaryotes, genome mutations may result in the formation of micronuclei, which are a consequence of chromosome fragmentation or malfunction of the mitotic apparatus. Thus, both clastogenic compounds and spindle poisons lead to an increase in the number of micronucleated cells. The micronucleus assay on *Xenopus laevis* is an International standard (ISO, 2006). One of the key functions of such biomarkers (micronucleus) is to provide an "early warning" signal of significant biological effects (changes at the genetic/molecular level) with sub-organism (molecular, biochemical and physiological) responses preceding those occurring at higher levels of biological organization such as cellular, tissue, organ, whole-body levels and, in fine, at population level.

2.4.1. Larvae exposure

Sexually mature *Xenopus* laevis were provided by the Developmental Biology Department of Rennes University (France). The experimental exposure condition was performed according to the International Standard 21427-1 [61]. The amphibian larvae were exposed at 22 ± 0.5 °C in reconstituted water (distilled tap water to which nutritive salts were added [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]). *Xenopus* exposure began on larvae at stage 50 of the *Xenopus* development table [57], characterized by the hind limb bud longer than broad, constricted at the base. For a given experiment, the larvae were taken from the same batch to reduce inter-animal genetic variability within each experiment. Larvae were exposed in groups of 15–20 animals (100 mL/larva) in 5 L glass flasks containing either the control medium (negative and positive controls) or the test medium containing Cd^{2+} as $Cd(NO_3)_2$ (99%, provided by Sigma–Aldrich) at 0.02, 0.2, 2, 5, 10 and 50 mg Cd^{2+}/L. For immobilized cadmium on ashes toxicity evaluation, MBM-LA concentration was 0.1 g/L. The negative control (NC) was the reconstituted water alone. The positive control was cyclophosphamide (CP, [6055-19-2], Sigma France) in reconstituted water at 20 mg/L [62]. CP is a standard indirect mutagen requiring metabolic activation in liver prior to becoming effective. Positive control was systematically performed in each experiment to check the responsiveness of the amphibian larvae. MBM-LA control (NC ashes) containing 0.1 g/L of ashes in reconstituted water was introduced in order to evaluate the innocuity of MBM-LA ashes alone. The larvae were submitted to a natural dark cycle at 22.0 °C ± 0.5 °C during the 12 days of exposure. They were fed every day on dehydrated aquarium fish food.

2.4.2. Acute toxicity

Acute toxicity (mortality or abnormal behaviour) of larvae was examined for 12 days according to the standardized recommendations [61] by visual inspection and comparison to negative control (NC). Abnormal behaviour corresponds to reduced and/or stopped growth of larvae, reduced food intake and abnormal mobility.

2.4.3. Micronucleus test, Genotoxicity

At the end of exposure, a blood sample was obtained from each anaesthetized larva (MS 222, Sandoz, France) by cardiac puncture with heparinized micropipettes (20% solution at 5000 IU/ml, Sigma, France). After fixing in methanol and staining with hematoxylin (Sigma, France), 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. [58] and consists of determining the theoretical medians of samples of size n (where $n \geq 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times \mathrm{IQR}/\sqrt{n}$, 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.

2.4.4. Cd analysis in xenopus's liver

At the end of the exposure, livers were dissected from each anaesthetized and punctured larva. Excess moisture was removed on absorbent paper, and the livers were pooled per test condition. Each pool was weighted (fresh weight). Samples were digested by nitric acid (5 μl of pure HNO_3/mg of liver) in closed borosilicate glass tubes at 100 °C for 2 h. After dilution of the digestates with 20 ml ultrapure water (MilliQ plus), Cd^{2+} concentrations were measured by atomic absorption spectrophotometry, with electrothermal atomization (PerkinElmer, SIMA 6000) according to experimental conditions reported in "Elemental analysis part". Each sample was analysed three times. Similar digestions were realised with pure nitric acid in order to ensure that glass vessel did not released Cd ions. Another experiment was realised with a mixture of pure nitric acid and 0.1 mL of Cd certified standard solution in order to confirm that Cd is not lost during the process. Certificated aqueous standards were obtained from Aldrich. The detection limit was 0.1 μg Cd^{2+} L^{-1}. The results are expressed in average metal concentrations accumulated in liver (3 replicates per exposure condition) in μg Cd^{2+} g^{-1} liver (fresh weight) ± SE (standard error). Statistical analysis used the software Sigma Stat 3.1. The kruskall-Wallis test was used to compare all the conditions versus the NC group. It was followed by Dunn's test for multiple comparisons versus a control group.

3. Preliminary chemical investigation – results and discussion

3.1. MBM ashes characterization

Characterization of ashes used in this work has been published in a previous paper [12], and the main results are reported here. Chemical analysis of major elements shows that both ashes present a similar composition (Table 1) with high calcium and phosphorus contents, up to 47% (weight). Considering that phosphorus is present as phosphate ions, the total amount of calcium and phosphate reach 82.7% and 77.3% for MBM-LA and MBM-BA, respectively. Other elements (Si, Al, Mg, Fe Na, K, SO_4^{2-} and Cl^-) make up the rest: 8% for MBM-LA and 13% for MBM-BA. The slight

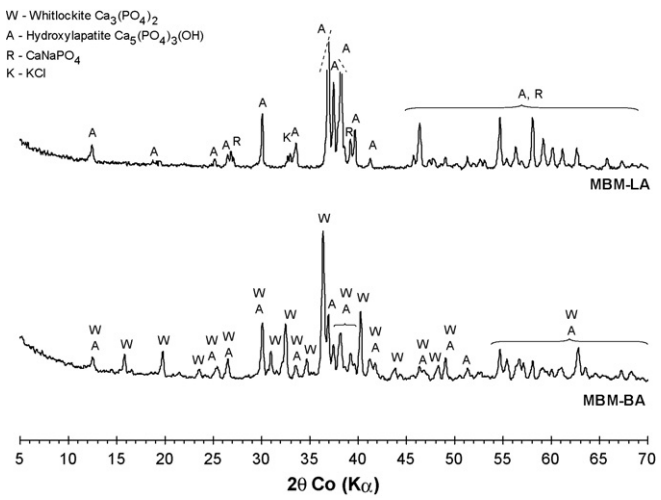

Fig. 1. XRD pattern of MBM-BA and MBM-LA.

difference may be explained by the fact that industrial ashes are co-incinerated with other wastes. Traces elements (such as Ba, Cr, Cu, Ni, Sr, Ti, V, Zn, etc.) are lower than 0.3%. Heavy metal contents of MBM-BA are much higher than in MBM-LA but remain of the same order of magnitude as those found in natural phosphate rocks [12].

XRD pattern (Fig. 1) and mineralogical compositions obtained by Rietveld analysis (Table 2) show that industrial ashes are mainly composed of whitlockite and HAP whereas laboratory ashes are only composed of HAP. This difference may be explained by the various combustion process used: incineration or co-incineration, operating temperature of incineration. Indeed, many authors [8,62–66] reported the formation of whitlockite/apatite mixtures when heating non-stoichiometric hydroxylapatite above 800 °C.

Previous to biological studies, a leaching experiment was performed according to European standard EN12–457 (solid/liquid ratio was 1/10; demineralised water was used as extraction medium and extraction time was 24 h) [14,15]. Table 3 reports the concentrations of leached elements in regard to European waste acceptance criteria for waste landfills [14,15]. The leachate concentrations (in μg of element per kg of ash) are quite low compared to the total amount of elements initially present in the ashes. Moreover, MBM-LA released much less heavy metals. According to the thresholds values given by European regulations [14,15] regarding the classification of waste to be landfilled, MBM-LA would be classified as an inert waste whereas MBM-BA could be considered as a non dangerous waste. Indeed some chrome pollution is observed on MBM-BA and it is probably coming from co-incinerated materials.

Table 2
Mineralogical composition of MBM-BA and MBM-LA obtained by Rietveld method (wt.%).

Mineral	HAP $Ca_5(PO_4)_2OH$	Whitlockite $Ca_3(PO_4)_2$	Rhenanite ($CaNaPO_4$)	KCl
MBM-BA	32	68	–	–
MBM-LA	91	–	8	1

3.2. Kinetic and mechanism of cadmium removal by MBM ashes

Cadmium uptake efficiency by MBM ashes in aqueous media was first evaluated by kinetic experiments. Cadmium concentrations in solutions were determined at different times with 25, 50, 100, 300, 1000 and 1500 mg/L starting solutions and ashes concentration of 2.5 g/L. Solutions were prepared with $Cd(NO_3)_2$, in distilled water, as Ma et al. showed that nitrate anions do not interfere with the apatite cation exchange reaction [67].

Fig. 2 represents pH evolution during cadmium uptake by MBM ashes for Cd^{2+} initial concentration of 25–1500 mg/L. The addition of MBM ashes onto a solution containing Cd^{2+} causes an immediate pH increase (Fig. 2). This first step may be explained by basic compounds dissolution contained in ashes probably induced by metals oxides such as CaO produced by combustion. In a second step, pH decreases to an equilibrium value depending on initial cadmium concentration in solution. After 45 h (2700 min), with 100 mg/L initial Cd^{2+} concentration solution, pH stabilized around 7 for MBM-BA and 9 for MBM-LA, indicating that MBM-LA basicity is higher. Thus, in order to compare Cd^{2+} uptake quantity by both ashes, pH was regulated by nitric acid addition for the concentrations of 50 and 300 mg/L for the tests involving MBM-LA.

The shapes of the curves representing Cd^{2+} uptake by gram of ashes versus time, for various initial Cd^{2+} concentration, highlights a mechanism involving at least two successive steps (Fig. 3). The first portion indicates that a rapid uptake occurs during the first hours. Then, the metal uptake slowly tends, after 45 h (2700 min), to reach either an equilibrium or a kinetically slow metal uptake. Some authors report indeed that equilibrium is not reached even after 10 days with synthetic apatite [39]. It can be noticed that metal uptake by ashes is dependent of initial Cd^{2+} concentration in solution. These results are in agreement with previous studies on the mechanism of Cd^{2+} uptake by synthetic HAP [28,33–35,41,44].

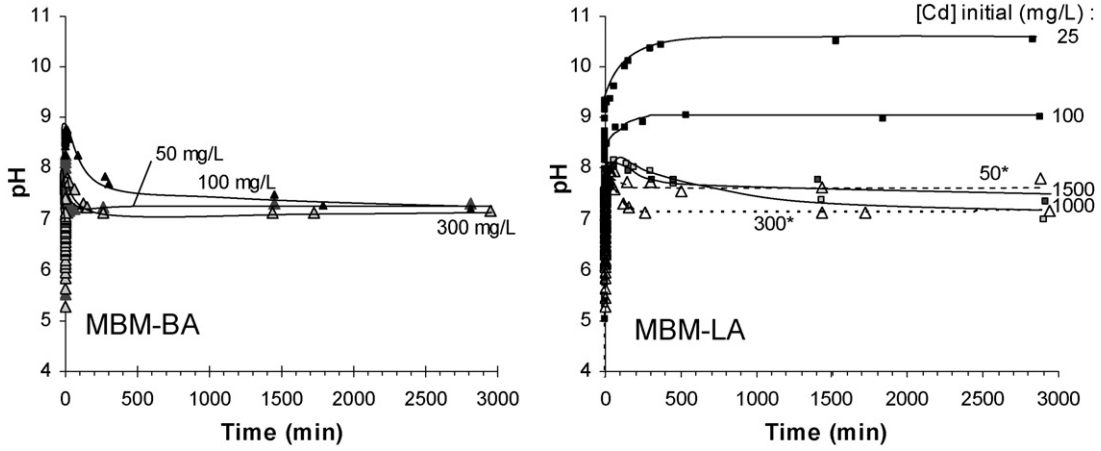

Fig. 2. pH evolution during cadmium uptake by MBM-BA and MBM-LA for different Cd^{2+} initial concentration (25–1500 mg/L). pH regulated for the concentrations of 50 and 300 mg/L(*).

Table 3
Leached elements (EN 12–457) by MBM-BA and MBM-LA (μg/kg), compared to European waste acceptance criteria in landfill.

	Waste acceptance criteria [14,15]			MBM ashes			
	Inert (i)	Non-hazardous (nh)	Hazardous (h)	MBM-BA		MBM-LA	
As (μg/kg)	500	2000	25 000	3	i	1	i
Ba (μg/kg)	20 000	100 000	300 000	999	i	34	i
Cd (μg/kg)	40	1 000	5000	8	i	0.1	i
Cr (μg/kg)	500	10 000	70 000	1104	nh	146	i
Cu (μg/kg)	2000	50 000	100 000	185	i	1	i
Ni (μg/kg)	400	10 000	40 000	58	i	1	i
Pb (μg/kg)	500	10 000	50 000	38	i	<1.2	i
Sb (μg/kg)	60	700	5000	28	i	1	i
Zn (μg/kg)	4000	50 000	200 000	523	i	<28	i

One can also notice that metal uptake quantity by MBM-LA is at least two times higher than that of MBM-BA. This difference may be explained by the presence of more soluble metal in MBM-BA as observed by leaching experiments [12]. In fact, these leached metals may compete with Cd^{2+} ions decreasing both Cd^{2+} uptake kinetic and capacity [68]. Moreover, it is generally agreed that sorption capacity increases with the specific surface area [34,43] and BET measurement shows that specific surface of MBM-BA (1 m^2/g) is lower than MBM-LA (4 m^2/g).

The above results indicate that the maximum Cd^{2+} uptake by MBM-LA observed is 57 mg Cd^{2+}/g (one can expect slightly higher Cd^{2+} uptake value with higher initial concentration cadmium). Comparison of this value with published results [25,27,29–33,35,36,38–44] is illustrated on Fig. 4. It is generally agreed that Cd^{2+} uptake by CaHAP is strongly dependent on CaHAP structure (crystallinity, specific surface area ...), CaHAP composition (fluorine, carbonate ... substitution in HAP) and experimental conditions (temperature, pH, aqueous solution composition ...). However Cd^{2+} sorption capacity is found to mainly depend on CaHAP specific surface area (BET). In fact, if experimental conditions are slightly different - particle size distribution, contact time (between 24 and 72 h), pH (between 4 and 6), and crystallinity of CaHAP - this figure tends to highlight a correlation between Cd^{2+} uptake quantity and BET surface area for synthetic HAP. Cadmium uptake by MBM-BA is in agreement to expected result. However, one can notice that MBM-LA quantity of Cd^{2+} uptake is higher than for synthetic HAP with similar specific surface area.

To go a little further in the mechanism description, XRD analyses of MBM-LA and MBM-BA before and after Cd^{2+} immobilization have been realized (Fig. 5). According to literature, the interaction mechanism of Cd^{2+} ions with CaHAP could be explained by three mechanisms, similar to those involved in lead removal by CaHAP: surface adsorption of Cd^{2+} [32,34,35,39,41,43,44,46], CaHAP dissolution followed by CdHAP precipitation [30–34,39,41] or ions exchange [28,33,35,40,42,44,47]. For some authors, Cd^{2+} sorption by HAP involves all three mechanisms [35,38,43,47,48]. Recently, Marchat et al. [32] proposed a two steps mechanism (Ca^{2+} ions substitution by Cd^{2+} at particle surface followed by their incorporation in the hydroxylapatite bulk) resulting in the formation of an apatite solid solution, $Ca_{10-x}Cd_x(PO_4)_6(OH)_2$, (CaCdHAP), which exhibits relatively low solubility allowing immobilization of cadmium. In the case of industrial ashes (MBM-BA), no major modification was observed and composition remained around 32% of CaHAP and 68% of whitlockite, according to Rietveld analysis. This could be due to the fact that Cd^{2+} interaction with CaHAP gives an apatite solid solution [32,34,35,39], $Ca_{10-x}Cd_x(PO_4)_6(OH)_2$, having an XRD pattern similar to the one of HAP [69].

The mechanism seemed to be different with laboratory ashes (MBM-LA) since new crystalline phases were observed after contact with Cd^{2+} solution. Kinetic experiments have shown that metal uptake by MBM-LA after incubation in 1500 ppm Cd solution during 48 h reach 57.3 mg/g. Rietveld analysis of Cd loaded ashes shows that MBM-LA contained nearly 95% of HAP, 3% of otavite, $Cd(CO_3)$, and 2% of rhenanite, $CaNaPO_4$. If we consider 1 g of MBM-LA, 3% of otavite represent 30 mg of $Cd(CO_3)$ that is to say 20 mg of Cd. MBM-LA containing 57.3 mg/g of cadmium, 20 mg/g are immobilized as otavite and then 37.3 mg/g as $Ca_{10-x}Cd_x(PO_4)_6(OH)_2$. So 35% of cadmium precipitated as $Cd(CO_3)$ whereas the remaining 65% were inserted in $Ca_{10-x}Cd_x(PO_4)_6(OH)_2$. The explanation for the formation of otavite could be the carbonates contained in MBM-LA, as observed in infrared analysis and evaluated at 6%

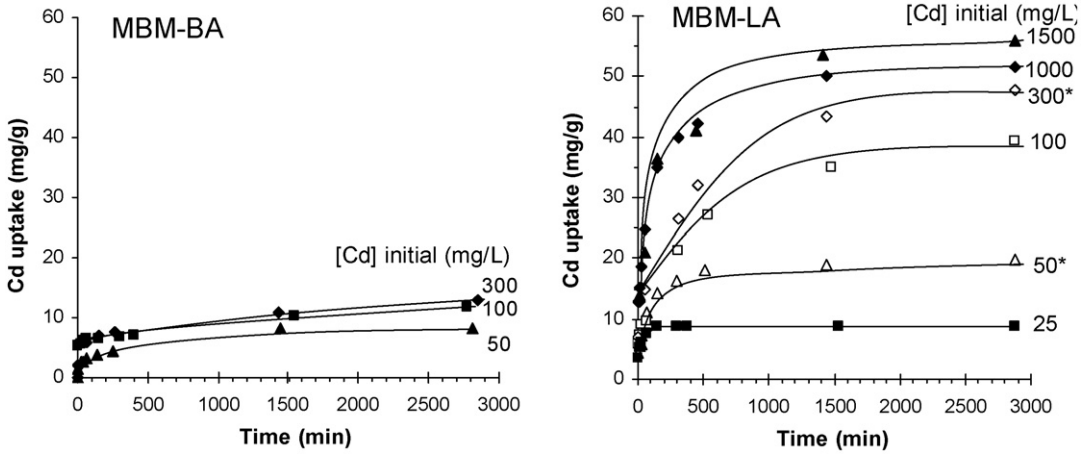

Fig. 3. Cadmium uptake (mg/g) by MBM-BA and MBM-LA for different Cd^{2+} initial concentration (25 to 1500 mg/L). pH regulated for the concentrations of 50 and 300 mg/L(*).

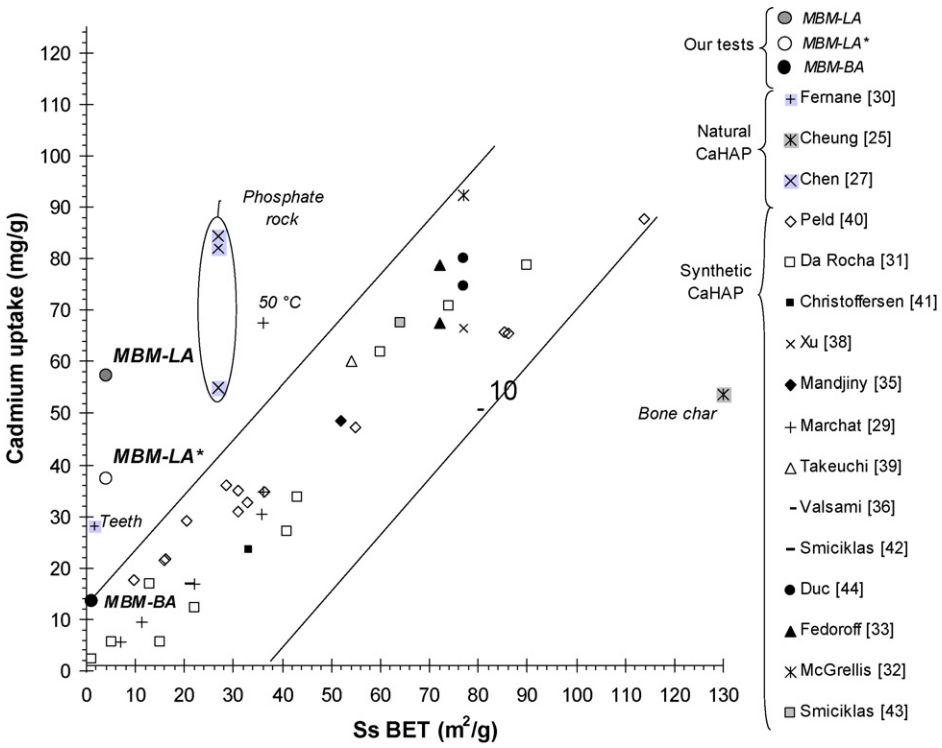

Fig. 4. Cadmium uptake quantity by MBM ashes compared to synthetic and natural HAP according to BET specific surface area (MBM-LA* represents total cadmium uptake by MBM-LA minus cadmium immobilized as otavite precipitation).

by calcimeter quantification. The formation of $Cd(CO_3)$ may contribute also to explain its higher cadmium capacity compared to MBM-BA, or to synthetic HAP (Fig. 4). Indeed, regarding only Cd^{2+} uptake relative to the $Ca_{10-x}Cd_x(PO_4)_6(OH)_2$ phase, i.e. MBM-LA* on Fig. 4, we obtained a value closer to the expected one.

At last, the ability of ashes to immobilize cadmium after its uptake Cd^{2+} was evaluated by leaching experiments. Analysis of the solutions underline ashes efficiency as Cd^{2+} released remains lower than 1% of Cd^{2+} uptake by both ashes after 24 h (final pH around neutrality).

These results underline ashes efficiency to uptake and sequestered Cd^{2+} and tend to prove the unavailability of Cd^{2+}. However, assessments of Cd^{2+} toxicity after immobilization on ashes remains necessary and essential to ensure whether ashes can be efficiently used for *in-situ* remediation of cadmium-contaminated aqueous solutions.

4. Toxicity and genotoxicity investigation – results and discussion

The above results demonstrate that MBM-LA presented a higher Cd^{2+} uptake quantity than MBM-BA. Moreover, MBM-LA harmlessness towards *Xenopus* larvae has been demonstrated for concentrations up to 5 g/L in a previous study (no acute toxicity and nor genotoxicity were observed when MBM-LA is used alone)

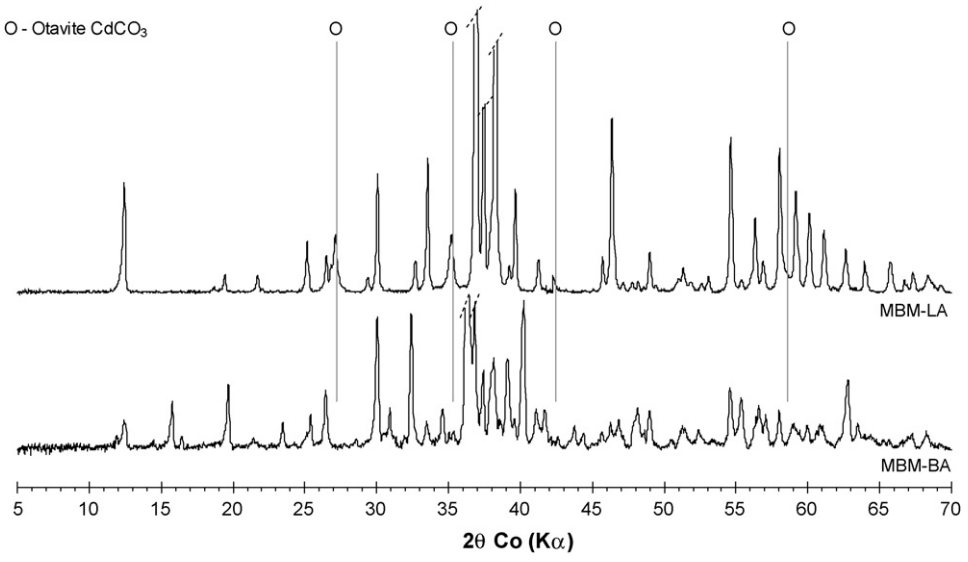

Fig. 5. XRD pattern of MBM-BA and MBM-LA after incubation with cadmium-contaminated solution.

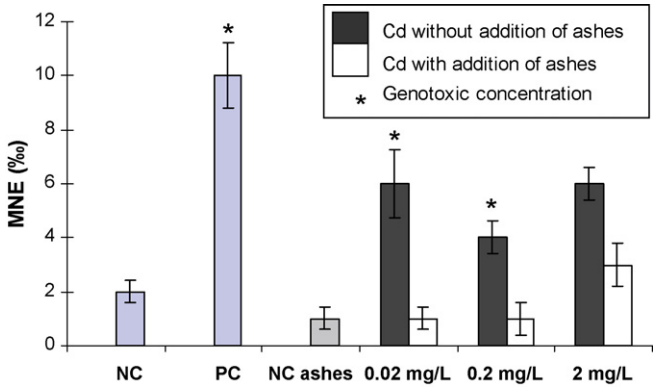

Fig. 6. Median value of micronucleated erythrocytes per thousand (MNE $^{\circ}/_{oo}$) values after 12 days of exposure of *Xenopus* larvae to different conditions: positive control (PC), negative control (NC), negative control with 0.1 g MBM-LA per L (NC ashes) and with various Cd concentrations (0.02, 0.2 and 2 mg/L). $n = 20$ larvae per concentration. * Indicates that genotoxicity is significantly observed according to the McGill test [58]. MNE $^{\circ}/_{oo}$ is expressed by the value of the median and the 95% confidence limits.

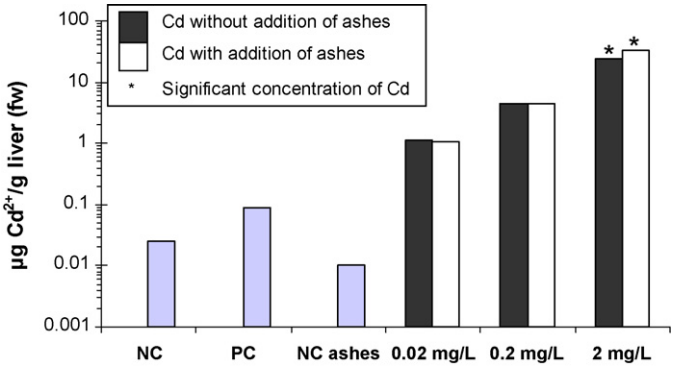

Fig. 7. Cd-bioaccumulated in *Xenopus* larvae livers exposed to Cd (0.02, 0.2 and 2 mg/L) with or without ashes for 12 days: negative control (NC), negative control with 0.1 g of MBM-LA/L (NC ashes), positive control (PC), fw: fresh weight. $n = 20$ livers. The results are expressed in average metal concentrations accumulated in liver (3 replicates per exposure condition) in µg Cd^{2+} g^{-1} liver (wet wt) ± SE (standard error). *: Significant bioaccumulation of Cd in livers of larvae compared to NC (Kruskall-Wallis followed by Dunn's test, $p < 0.05$).

[10]. So, biological experiments were carried out with MBM-LA and Cd^{2+}.

4.1. Evaluation of cadmium toxicity and genotoxicity on Xenopus larvae

Amphibian larvae have been exposed for 12 days to various cadmium concentrations: 0.02, 0.2, 2, 5, 10, and 15 mg/L. Acute toxicity appeared at 2, 5, 10 and 15 mg/L of Cd^{2+}, and the lethality rates were 27%, 40% and 100% for 5, 10 and 15 mg/L cadmium concentration, respectively. Moreover, amongst surviving larvae physical signs of anaemia, lower size, restricted food-intake or abnormal behaviour (perturbed swimming are apparent). With lower concentrations no sign of acute toxicity was observed.

Regarding genotoxicity assessment, cadmium concentrations were limited to 0.02, 0.2 and 2 mg/L because of the mortality observed at higher doses. Micronucleus test results (Fig. 6) revealed cadmium genotoxicity on larvae for all concentrations without ashes. The negative control led to a MNE $^{\circ}/_{oo}$ (number of micronucleated erythrocytes per thousand) value equal to 2 $^{\circ}/_{oo}$ whereas significantly higher MNE were observed with cadmium: MNE of 6, 4 and 6 $^{\circ}/_{oo}$, for 0.02, 0.2 and 2 mg/L of Cd^{2+}, respectively.

4.2. Evaluation of toxicity and genotoxicity of immobilized Cadmium by MBM-LA on Xenopus larvae

The above data underline the strong cadmium acute toxicity and genotoxicity towards *Xenopus* larvae. Evaluation of cadmium toxicity after sequestration on ashes matrix is necessary and essential to know whether ashes can be safely used to remove Cd^{2+} from wastewater.

Amphibian larvae were exposed during 12 days to cadmium (to six cadmium concentrations: 0.02, 0.2, 2, 5, 10 and 50 mg/L) after a 20 h contact time of the initial cadmium solutions with ashes (100 mg/L). Acute toxicity was observed at 5, 10 and 50 mg/L, and the lethality rates were 33%, 67% and 100%, respectively. These results were similar to those observed with cadmium only. However, no toxicity was observed at concentrations equal and lower than 2 mg/L. As seen previously, 2 mg/L of Cd^{2+} alone (without MBM-LA addition) induced acute toxicity; this first result revealed some efficiency of ashes to inhibit cadmium toxicity.

Genotoxicity assessment of cadmium solutions, with and without MBM-LA, is presented on Fig. 6. As expected [10], addition of 0.1 g/L of ashes in aqueous media (negative control ashes) did not induce any genotoxicity. Moreover, although Cd^{2+} alone induced genotoxicity in larvae when exposed to 0.02, 0.2 or 2 mg Cd^{2+}/L, the addition of only 0.1 g of MBM-LA totally inhibited genotoxicity expression in larvae. Indeed, MNE $^{\circ}/_{oo}$ values with ashes (1–3 $^{\circ}/_{oo}$) were significantly lower than MNE $^{\circ}/_{oo}$ values without ashes (3–6 $^{\circ}/_{oo}$). It can also be noted that the number of micronucleated erythrocytes per thousand of larvae incubated with 0.02 and 0.2 mg/L Cd^{2+} with MBM-LA remained similar to negative standard (MNE = 2 $^{\circ}/_{oo}$).

Furthermore, cadmium bioaccumulation evaluation was realized by measuring Cd^{2+} concentration in *Xenopus* larvae's liver at the end of the exposure. These results (µg of Cd per g of liver) versus Cd^{2+} exposure solution concentration are reported on Fig. 7. The Cd^{2+} concentration in liver increased dramatically with Cd^{2+} concentration in solution and there was no significant difference for Cd^{2+} concentration in liver between experiments realized with and without MBM-LA. Moreover, comparison of these values and genotoxic results seemed somewhat unexpected as 20 mg Cd^{2+}/g of liver with MBM-LA (2 mg/L Cd^{2+} solution) was not genotoxic, whereas 2 mg Cd^{2+}/g of liver without MBM-LA (0.02 mg/L Cd^{2+} solution) was genotoxic. These results tend to show that there is no direct correlation between the concentration of Cd^{2+} in liver and the genotoxicity. However, it can be pointed out that the expression of toxicity in organisms depends on several complex mechanisms which may act as antagonistic and/or synergistic such as repair mechanisms and genetic damage, concentrations, chemical Cd-speciation, etc.

5. Conclusion

Kinetic and mechanistic studies on both industrial (MBM-BA) and laboratory (MBM-LA) meat and bone meal combustion residues demonstrate their efficiency to remove Cd^{2+} from aqueous solution. After 45 h, with 1500 mg/L initial Cd^{2+} solution, MBM-LA capacity reaches nearly 57 mg of Cd^{2+}/g of ashes whereas the metal uptake quantity of MBM-BA is at least two times lower. This result may be partly explained by the lower specific area of MBM-BA compared to MBM-LA (1 and 4 m^2/g, respectively). Moreover, Rietveld analysis of XRD pattern of MBM-LA ashes, after contact in Cd^{2+} contaminated solutions, reveals the formation of $Cd(CO_3)$ which possibly increases the Cd^{2+} uptake. Leaching experiments at pH 3 and 13 with a solid/liquid ratio of 1/400 shows that Cd^{2+} release is lower than 1% of Cd^{2+} uptake by both ashes.

Toxicological studies on *Xenopus* larvae underline ashes efficiency to immobilize cadmium in solution and to decrease or inhibit its toxicity. Indeed, if Cd^{2+} solutions at 0.02, 0.2 and 2 mg Cd^{2+}/L

induce genotoxicity in amphibian larvae, addition of only 0.1 g MBM-LA/L allows inhibiting or limiting these genotoxic effects. However, Cd^{2+} bioaccumulation in larvae's liver is similar for both experiments, with and without ashes. Indeed, for similar concentration of cadmium in liver, genotoxicity expression is sometimes different according to considered cadmium alone or with ashes.

Even if all results tend to show the efficiency of meat and bone meal combustion residue for remediation of cadmium-contaminated aqueous media, additional studies should be carried out to fully understand complex biological mechanisms involved in cadmium toxicity.

Acknowledgement

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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