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# Assessment of lead ecotoxicity in water using the amphibian larvae (*Xenopus laevis*) and preliminary study of its immobilization in meat and bone meal combustion residues

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**Abstract**

Lead (Pb) is a major chemical pollutant of the environment. It has been associated with human activities for the last 6000 years. Quite rightly, it remains a public health concern today. The present investigation evaluates the toxic potential of Pb in larvae of the toad *Xenopus laevis* after 12 days exposure in lab conditions. Acute toxicity, genotoxicity and Pb bioaccumulation were analyzed. The genotoxic effects were analyzed in the circulating blood from the levels of micronucleus induction according to the French standard micronucleus assay (AFNOR 2000 Association française de normalization. Norme NFT 90-325. Qualité de l'Eau. Evaluation de la génotoxicité au moyen de larves d'amphibien (*Xenopus laevis*, *Pleurodeles waltl*)). Lead bioaccumulation was analyzed in the liver of larvae at the end of exposure. Moreover, the toxic potential of lead, in aquatic media, was investigated in the presence of meat and bone meal combustion residues (MBMCR) known to be rich in phosphates and a potential immobiliser of lead. Previously, acute toxicity and genotoxicity of MBMCR alone were evaluated using *Xenopus* larvae. The results obtained in the present study demonstrated: (i) that lead is acutely toxic and genotoxic to amphibian larvae from 1 mg Pb/l and its bioaccumulation is significant in the liver of larvae from the lowest concentration of exposure (1 $\mu$g Pb/l), (ii) MBMCR were not acutely toxic nor genotoxic in *Xenopus* larvae, (iii) lead in presence of MBMCR induced inhibition or reduction of the toxic and genotoxic potential of lead in water at concentrations that do not exceed the capacity of MBMCR of Pb-binding (iv) Pb accumulation in larvae exposed to lead with MBMCR in water was lower than Pb-accumulation in larvae exposed to lead alone except at the concentration of 0.01 mg Pb/l suggesting complex mechanisms of MBMCR interaction in organisms. The results confirm the high toxicity and genotoxicity of lead in the aquatic compartment and suggest the potential utility of MBMCR for use in remediation.

## Introduction

One pollutant that has received considerable attention is lead (Pb), a hazardous heavy metal. It has been employed in the past to manufacture of drain pipes, cookery pots, weapons and machinery (Vogiatzis & Loumbourdis 2001). More recently, it has been employed in many

varieties of industrial applications and products such as storage batteries, chemicals, pigments, paints, gasoline (Moore & Ramamoorthy 1984; Landrigan 1990), in mining and smelting activities, and has been spread on agricultural land for instance in sewage sludge (Vogiatzis & Loumbourdis 2001). Massive lead utilization has resulted in extensive lead contamination of the environment.

Although the quantities of lead released have been significantly limited since the introduction of unleaded gasoline, its persistence in ecosystems and its adverse effects on living organisms are still a major concern. The toxicological effects of lead are extensively documented in many physiological systems notably in man (Ter Haar 1981; Kopp *et al*. 1988; Landrigan 1990; Verity 1990; Beck, 1992; Goyer 1993; Papanikolaou *et al*. 2005) but amphibians have also been particularly well investigated (Perez-Coll *et al*. 1988; Pérez-Coll & Herkovits 1990; Steele *et al*. 1991; Strickler-Shaw & Taylor 1991; Sobotka & Rahwan 1995; Stansley & Roscoe 1996; Stansley *et al*. 1997; Kumar 1999; Rice *et al*. 2001; Rosenberg *et al*. 2003; Fink & Salibian 2005). However, the vast majority of the studies devoted to the impact of lead in amphibians have been oriented towards toxicity, physiology, biochemistry and immunology in embryos or adults and very little or no information is currently available on the genotoxic effects of lead, especially in larvae. However, the presence of genotoxic pollutants in water can have repercussions on aquatic species, but also on non-aquatic species, via food chains, or simply as a result of using the water for drinking. One should therefore be aware of the hidden risks stemming from potentially genotoxic substances in the aquatic environment. The interaction of genotoxic compounds with DNA initially causes structural changes in the DNA molecule. Unrepaired damage can potentiate other cell lesions and thus lead to tumour formation (Vuillaume 1987; Malins *et al*. 1990). Hence, the genotoxic risk of pollutants must absolutely be evaluated.

A variety of strategies has been employed to determine the effect of environmental pollutants on amphibians: organisms placed in the field (Cooke 1981), organisms exposed in the lab to media harvested from field sites (Burkhart *et al*. 1998; Gauthier *et al*. 2004; Mouchet *et al*. 2005a, b, 2006) or organisms exposed in the lab to controlled laboratory conditions (Pérez-Coll & Herkovits 1990; Sobotka & Rahwan 1995; Mouchet *et al*. 2005b). In the present investigation, amphibian larvae were exposed to lead in controlled lab conditions in order to evaluate three different endpoints after 12 days of exposure according to the French standard micronucleus assay (AFNOR 2000): (i) acute toxicity of larvae (ii) genotoxicity as the expression of the clastogenic and/or aneugenic effects of lead in the erythrocytes of the circulating blood (iii) and Pb accumulation in the liver. Moreover, in order to open the way towards Pb remediation strategies in the aquatic environment, Meat and Bone Meal Combustion Residues (MBMCR), which are natural low cost apatite-rich substances, were analyzed alone and in the presence of lead because of their high Pb-binding capacities (Deydier *et al*. 2003, 2005). The possibility of using MBMCR, to sequester lead from aqueous media, as substitute for the rather costly hydroxyapatite, was also investigated. The same three biological endpoints were investigated in amphibian larvae exposed to lead with or without MBMCR in their water, to evaluate the possible immobilization of lead by MBMCR and to examine the possible use of MBMCR materials in remediation of Pb-contaminated sites.

The *Xenopus laevis* model was chosen because of its ecotoxicological relevance which has already been well established in a large array of studies focused on biomarkers such as induction of biotransformation enzyme activities (Békaert *et al*. 2002; Gauthier *et al*. 2004), micronucleus (Ferrier *et al*. 1998), primary DNA damage (comet) (Mouchet *et al*. 2005a, b, 2006), adduct formation (Békaert *et al*. 2002), and teratogenic malformations with the FETAX assay (Chenon *et al*. 2002; Prati *et al*. 2002).

## Materials and methods

### *MBMCR*

Meat and Bone Meal (MBM) were sterilized at 133 °C/3 bars for 20 min to inactivate any bovine spongiform encephalopathy protein. The MBM was then burned by two successive calcinations in an electric furnace programmed to reach 850 °C at 2 °C/min. The residues were washed with ultra

pure water, and the pH adjusted to 7 with nitric acid, before filtration (250 $\mu$m pore size) to remove all water soluble residues. The composition of such Meat and Bone Meal Combustion Residues (MBMCR) was determined by chemical analysis to be (Deydier *et al*. 2005): water (0.23%); phosphates (55%); calcium (28%); phosphorus (18%); sodium (3%); potassium (3%); sulfur (1.78%); magnesium (0.95%); iron (0.69%); zinc (0.08%); copper (0.02%); aluminum (0.21%); silica (0.02%).

*Xenopus maintenance and breeding*

Sexually mature *Xenopus* were provided by the Developmental Biology Department of Paul Sabatier University (Toulouse, France). *Xenopus* males were injected with 400 IU of human chorionic gonadotropin (HCG) and females were injected with 700 IU of HCG to induce spawning. The males and females were then placed together in normal tap water at 22 ± 2 °C. Twelve hours later, the pair was separated and viable eggs were maintained in an aquarium also containing normal tap water at 20–22 °C, until they reached a development stage appropriate for experimentation (usually 3 weeks). The larvae were fed every day on dehydrated aquarium fish food. Each day, one third of the water was renewed before feeding.

*Exposure conditions*

The experimental exposure conditions are described in the French Standard AFNOR NF T90–325 (AFNOR 2000). AFNOR is the French National Organization for quality regulations. The water to which the amphibian models were exposed was reconstituted water (water distilled from tap water in 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] at 22 ± 0.5 °C). Exposure began on larvae at stage 50 of the *Xenopus* development table (Nieuwkoop & Faber 1956) characterized by the hind limb bud longer than broad, constricted at the base. For a given test, the larvae were taken from the same hatch to reduce inter-animal variability. They were exposed in groups of 15 or 20 (100 ml/larvae) in 5-l glass flasks containing either a control medium – negative, positive control and MBMCR control – or a test medium (different concentrations of lead, lead mixed with MBMCR and MBMCR alone). The negative control was the reconstituted water alone. The positive control was cyclophosphamide (CP, [6055-19-2], Sigma France) in reconstituted water at 20 mg/l (AFNOR 2000). The MBMCR control was 0.1 g/l of MBMCR in reconstituted water. For MBMCR analysis, the concentrations of MBMCR alone were: 0.1–0.5 to 2.5–5.0 g/l. For analysis of lead (as lead nitrate $Pb(NO_3)$, [10099-74-8] Sigma-Aldrich France, purity ≥99.0% 20 g Pb/l dissolved in ultra-pure water) and lead/MBMCR mixtures, two independent experiments (A and B) were conducted. In experiment A, the concentrations of lead were: 0.001, 0.01, 0.1, 1, 10, 30, 50 and 100 mg Pb/l. In experiment B, the concentrations of lead were 0.1, 1 and 10 mg Pb/l. The MBMCR concentrations in lead/MBMCR mixtures were 0.1 g/l in both experiments. For each condition, lead/MBMCR mixtures were first prepared by dilution in reconstituted water of the lead-concentrated solution, and then by addition of the MBMCR concentration in each lead condition and finally by shaking overnight the mixture. The larvae were submitted to a 12 h light: 12 h dark cycle. They were fed every day on dehydrated aquarium fish food. The 5-l flasks were partially immersed in water baths to maintain the water temperature at 22 ± 0.5 °C. The media in control and exposed flasks were renewed daily.

*Acute toxicity*

Acute toxicity to the larvae exposed to lead, lead/MBMCR mixture and MBMCR alone was examined for 12 days (AFNOR 2000) by visual inspection, compared to the negative control group: severe toxicity (death) and weak toxicity (abnormal behaviour, reduced size, diminished food intake, signs of anemia).

*Micronucleus test, genotoxicity assay*

For each experiment with (i) MBMCR alone, (ii) lead alone, (iii) lead in the presence of MBMCR (experiments A and B), 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 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. Since micronucleus frequency was not normally distributed, median values and quartiles were calculated instead of means with the statistical method described by McGill *et al*. (1978). For each group of animals, the results (number of micronucleated erythrocytes per thousand, MNE) obtained for each larva 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*. (1978) and consists in determining the theoretical medians of samples of size $n$ (where $n \geq 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times IQR/\sqrt{n}$, 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 negative control group is significant to within 95% certainty if there is no overlap.

*Pb analysis*

At the end of the exposure in experiments A and B, 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 weighed (wet wt). Samples were digested by nitric acid (5 $\mu$l of pure $HNO_3$/mg of liver) in closed borosilicate glass tubes at 100 °C for 2 h. After dilution of the digestates to 20 ml with ultrapure water (MilliQ plus), lead concentrations were measured by atomic absorption spectrophotometry, with electrothermal atomization (Perkin Elmer, SIMA 6000). Certificated aqueous standards were obtained from Aldrich. The detection limit was 0.1 $\mu$g Pb/l. The results are expressed in average metal concentrations accumulated in liver (3 replicates per exposure condition) in $\mu g\ Pb^{2+}/g$ liver (wet wt) ± SE (Standard Error). Statistical analysis used Sigma Stat 3.1. The Kruskall–Wallis test was used to compare all the conditions versus the negative control. It was followed by Dunn's test for multiple comparisons versus a control group. *T*-test was used to analyse dose effect relationships and the "ash-effect" relationship (comparison on the basis of two and two experimental conditions assuming normality and equal variance of data).

## Results

*Lead*

*Acute toxicity of lead*

Two independent experiments (A and B) were conducted.

Lead appeared to be severely toxic to *Xenopus* larvae exposed from 10 mg/l to 100 mg/l in experiment A (Table 1). Indeed, 5%, 30%, 87% and 100% of lethality were obtained at 10, 30, 50, 100 mg/l, respectively, at the end of exposure. Larvae exposed to 100 mg Pb/l died after 24 h of exposure. Larvae exposed to 50, 30 and 10 mg/l died in 48, 72 and 96 h, respectively. Surviving larvae at 10 and 30 mg Pb/l presented physical signs of anaemia, lower size and restricted food-uptake compared to the negative control group. Moreover, surviving larvae at 30 mg/l showed abnormal swimming behaviour which was largely perturbed. Lead was weakly toxic to larvae at 1 mg/l, since few signs of anaemia, diminished food intake and size were noted. No signs of acute toxicity were observed for *Xenopus* tadpoles exposed to lower concentrations (0.001, 0.01, 0.1 mg Pb/l).

*Table 1*. Acute toxicity to *Xenopus* larvae exposed to lead (mg/l) for 12 days in experiment A and B.

| | Lead concentration (mg/l) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0.001 | 0.01 | 0.1 | 1 | 10 | 30 | 50 | 100 |
| Experiment A | – | – | – | – | + | + (5%) | ++ (30%) | ++ (87%) | ++ (100%) |
| Experiment B | – | NE | NE | – | + | ++ (53%) | NE | NE | NE |

%: percentage of dead. NE: concentration not explored.– : no intoxication of larvae; +: weak intoxication; ++ severe intoxication.

In experiment B, lead was severely toxic to *Xenopus* larvae exposed to 10 mg/l, resulting in 53% of mortality (Table 1). Surviving larvae also presented strong physical signs of anaemia, lower size and restricted food intake compared to the negative control group. Moreover, abnormal behaviour showing perturbed swimming was observed. 1 mg Pb/l induced low toxicity since signs of slight anaemia, and diminished diet and size were recorded. 0.1 mg Pb/l did not induce any signs of acute toxicity.

*Genotoxicity of lead*

The genotoxicity of lead was independently evaluated in experiments A and B. The median value of MNE for negative control was $6 \pm 1.40$ in the experiment A and $6 \pm 0.87$ in the experiment B (Figure 1). Positive control of experiment A showed significantly higher MNE compared to the negative control group. No positive control in was carried out in experiment B.

Results of experiment A showed that 1 and 10 mg Pb/l lead to genotoxicity in erythrocytes of *Xenopus* larvae, resulting in 14 and 202 MNE, respectively. The lowest Pb concentrations (0.01 and 0.1 mg/l) were not genotoxic. MNE frequencies increased with increasing lead concentrations.

Results of experiment B (Figure 1) confirmed that 1 mg Pb/l induced a significant micronucleus induction, whereas 0.1 mg/l did not, compared to the negative control group.

*Pb analysis*

Bioaccumulation of lead was determined in livers. Only traces of lead were detected in controls of both experiments (NCA, NC, PC, Figure 2), while a significant Pb-bioaccumulation was observed for individuals exposed to lead, whatever the lead concentration, compared to the control group. Accumulation of lead ranged from $2.91 \pm 0.151$ to $114.05 \pm 1.48$ \$g Pb/g w.wt for larvae exposed to 0.01 from 30 mg Pb/l, respectively in experiment A. In experiment B, accumulation of lead ranged from $7.14 \pm 0.84$ to $1.42 \pm 0.58$ \$g Pb/g w.wt for larvae exposed to 0.1 and 1 mg Pb/l, respectively. Moreover, dose-dependent bioaccumulation was observed for Pb-treated individuals (Figure 2) in both experiments.

*MBMCR alone*

*Acute toxicity of MBMCR*

MBMCR were not toxic to amphibian larvae exposed to 0.1, 0.5, 2.5, 5 g/l (data not shown). The growth of larvae exposed to MBMCR was even faster than that of the negative control group. There was no difference in size and food intake, for the different MBMCR concentrations tested.

*Genotoxicity of MBMCR*

The micronucleus test results are presented in Figure 3. The median value of MNE for the negative control was $6 \pm 1.40$. The positive control showed significantly higher MNE than the negative control. MNT results reveal that MBMCR were not genotoxic to amphibian larvae, at any of the concentrations tested (0.1, 0.5, 2.5, 5 g/l). Moreover, median values of MNE related to MBMCR treatment were significantly lower than those of the negative control. No dose-effect

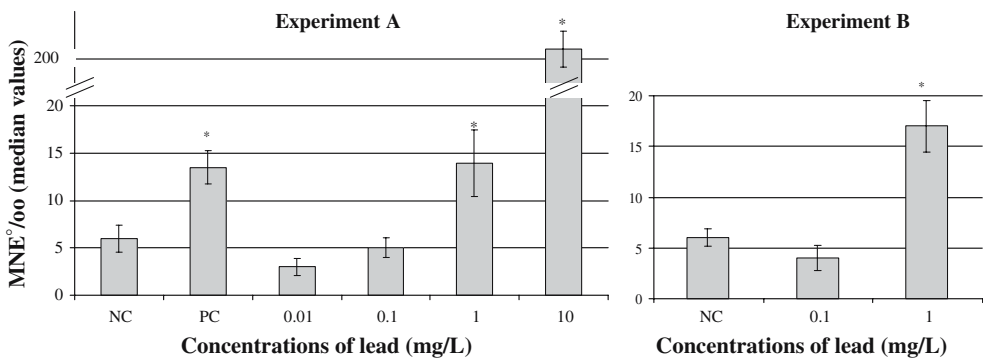


*Figure 1.* Results of the micronucleus assay in larvae exposed to lead (mg/l) for 12 days in the experiments A and B. NC: negative control, PC: positive control (cyclophosphamide, CP 20 mg/l). Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand, MNE) and their 95% confidence limits. *: genotoxic concentration compared to the negative control group.

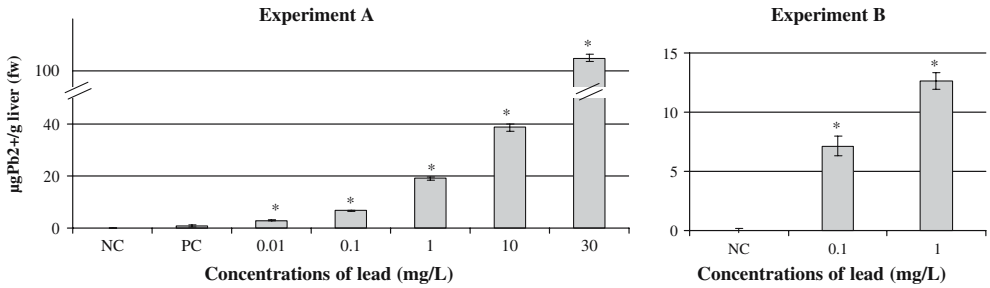


*Figure 2.* Bioaccumulation in *Xenopus* larvae exposed to lead (mg/l) for 12 days in experiments A and B. NC: negative control, PC: positive control (cyclophosphamide, CP 20 mg/l). Bioaccumulation is calculated in $\mu$g $Pb^{2+}$/g liver (fresh weight) and expressed as the values of the means ± SE. *: significant bioaccumulation of $Pb^{2+}$ in livers of larvae compared to NC (Kruskall-Wallis followed by Dunn's test, $p < 0.05$).

relationship between the different MBMCR concentrations was noted since 0.5, 2.5 and 5 g/l of MBMCR led to identical MNE.

## *LEAD with MBMCR (LEAD /MBMCR)*

### *Acute toxicity of lead in the presence of MBMCR*

Acute toxicity of lead/MBMCR mixtures was investigated in experiments A and B.

In experiment A, lead/MBMCR mixtures were not acutely toxic to *Xenopus* larvae (data not shown), irrespective of the lead concentration (0.01, 0.1, 1, 10 mg/l) except for the 30 mg/l concentration (10% of mortality). No difference was noted (based on size and food intake) between the non-toxic concentrations and the negative control group.

In experiment B, no signs of acute toxicity to *Xenopus* larvae were observed, whatever the lead concentration in the presence of MBMCR (0.1–1 and 10 mg Pb/l).

### *Genotoxicity of lead in the presence of MBMCR*

The genotoxicity of lead was independently evaluated in experiments A and B (Figure 4). MBMCR Negative control showed significantly lower MNE compared to the negative control group in both experiments. The results of experiment A showed that in the presence of MBMCR, none of the lead concentrations were genotoxic to *Xenopus* larvae, except 30 mg Pb/l which was genotoxic (median value:23.5) compared to the negative control. MNE values relative to 0.01, 0.1, 1 and 10 mg Pb/l with MBMCR were similar.

The results of experiment B (Figure 4) also showed that in the presence of MBMCR no lead concentrations were genotoxic to *Xenopus* larvae. With MBMCR, MNE values for 0.1, 1 and 10 mg Pb/l were similar.

### *Pb analysis*

In the presence of MBMCR, Pb-accumulation in experiment A (Figure 5) was not significant in

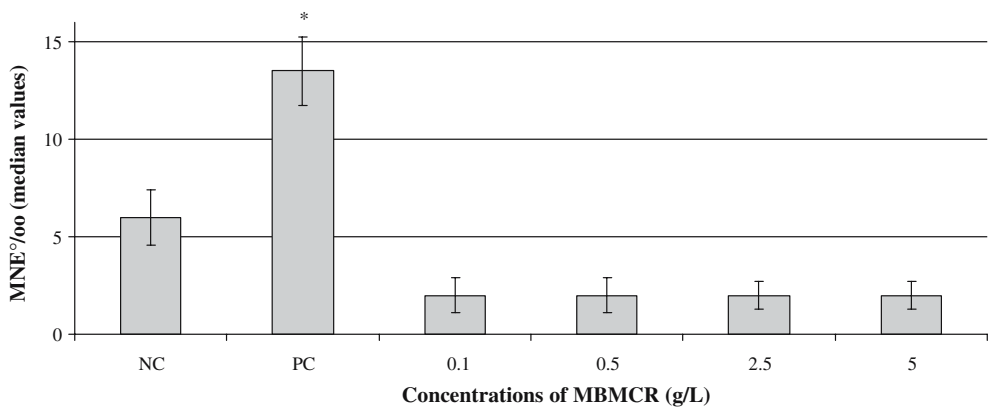


*Figure 3.* Results of the micronucleus assay in larvae exposed to MBMCR alone (g/l) for 12 days. NC: negative control, PC: positive control (cyclophosphamide, CP 20 mg/l). Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand, MNE) and their 95% confidence limits. *: genotoxic concentration compared to the negative control group.

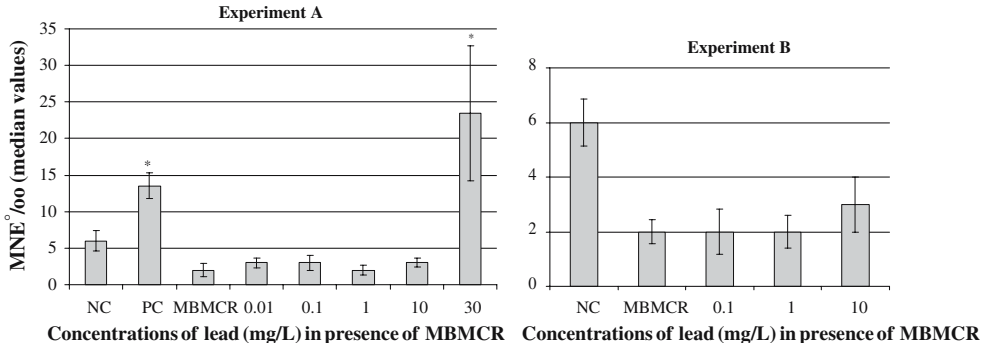


*Figure 4*. Results of the micronucleus assay in larvae exposed to lead in the presence of MBMCR for 12 days in experiments A and B. NC: negative control, PC: positive control (cyclophosphamide, CP 20 mg/l), MBMCR: meat and bone meal combustion residues control (0.1 g/l). Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand, MNE) and their 95% confidence limits. *: genotoxic concentration compared to the negative control group.

larvae exposed to 0.0, 0.1 and 1 mg/l, compared to the negative control, whereas it was significant in larvae exposed to 10 and 30 mg/l of Pb. In experiment B (Figure 5), Pb-accumulation was significant for all lead concentrations. Moreover, a dose-dependent bioaccumulation was observed for Pb-treated individuals with MBMCR in both experiments.For each given Pb-concentration, MBMCR induced lower significant Pb-accumulation except for 0.01 mg/l in experiment A since this concentration induced significantly higher Pb-accumulation in liver (*t*-test, $p < 0.001$).

## Discussion

### *Acute toxicity, genotoxicity and lead accumulation*

Acute toxicity of lead in *Xenopus* larvae was observed from 1 to 100 mg Pb/l. This is in agreement with the results of Khangarot and Ray (1987) who reported $LC_{50}$ values for tadpoles of *Rana hexadactyla* of 100, 66.7, 41.3 and 33.3 mg/l after 24, 48, 72 and 96 h, respectively. It is also in agreement with those of Stansley *et al*. (1997) who obtained high mortality in *R. palustris* tadpoles, exposed from the egg stage for 10 days to a total mean lead concentration of 1.670 and 0.944 mg Pb/l. In contrast, the same authors obtained no difference in mortality with *R. catesbeiana* among treatments ranging from 0.346 to 1.256 mg Pb/l as total lead mean. In the same way, Kumar (1999) obtained 10, 30, 40, 60, 90 and 100% of mortality of *R. tigrina* larvae exposed to 0.2, 0.4, 0.6, 0.8, 1 and 1.2 mg Pb/l, respectively. The evidence is that comparison must be carried out very carefully since many factors can interact in the final toxicity expression. Indeed, differences can be inherent to amphibian species. Differences can also be attributed to the exposure stage: in amphibians embryo stages can be more sensitive than larval stages

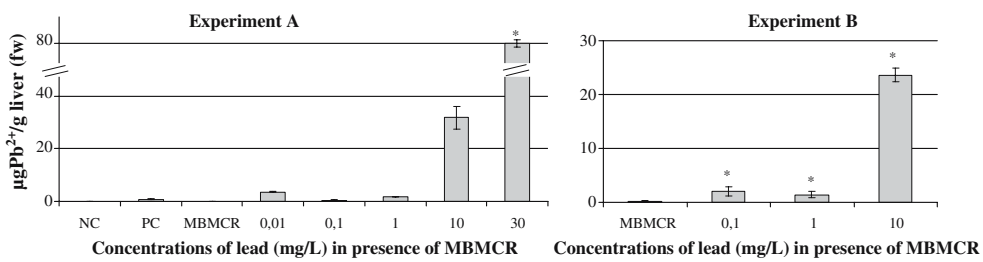


*Figure 5*. Pb-bioaccumulation in *Xenopus* larvae exposed to lead (mg/l) in the presence of MBMCR (0.1 g/l) for 12 days in experiments A and B. NC: negative control, PC: positive control (cyclophosphamide, CP 20 mg/l), MBMCR: meat and bone meal combustion residues control (0.1 g/l). Bioaccumulation is calculated in $\mu$g $Pb^{2+}$/g liver (fresh weight) and expressed as the values of the means ± SE. *: significant bioaccumulation of $Pb^{2+}$ in liver larvae compared to NC (Kruskall–Wallis followed by Dunn's test, $p < 0.05$).

which can be themselves more sensitive than adults to metal toxicity.

Moreover, acute toxicity is due to the fraction lead really available to the larvae, which was probably lower than the nominal concentration, since lead can be adsorbed onto the glass of the tanks or interact with ions (hydroxides, carbonates, sulfides, ...) or organic materials (food, ....) present in the water depending on the chemical conditions. For instance, the presence of other salts in the reconstituted water (see Materials and methods section, exposure conditions) could reduce the availability of lead to larvae. Another source of variability in the reconstituted water may come from the distilled water itself. For instance, the ionic composition of the distilled water used in experiments A and B was slightly different due to the use of two distinct apparatuses to produce the water. A difference of acute toxicity on *Xenopus* larvae between experiments A and B was noted and this could be explained by the variability between any two batches of eggs; indeed, the two experiments were carried out using larvae from two separate layings. Moreover, the physical conditions of the exposure media can also contribute to differences in toxicity expression such as water hardness etc. As mentioned above, the ionic water content may have influenced the final lead concentration available for the amphibian larvae in the water, explaining the differences in the toxicity results observed in experiments A and B.

The toxicological effects of lead on amphibians have been extensively documented, especially in embryos. For instance, in *Bufo arenarum*, lead induces deformities such as underdeveloped gills, sigmoidal body shape, and stunted tails at various stages of development (Pérez-Coll *et al*. 1988; Pérez-Coll & Herkovits 1990); in *Xenopus laevis*, it induces neural tube defects, tail curvature and lordoscoliosis (Sobotka & Rahwan 1995). The recent paper of Fink and Saliban (2005) summarizes the results of studies that examined the biochemical and physiological effects of Pb in an *in vivo* amphibian model (*B. arenarum*). In tadpoles, toxicological effects are also documented: in *Rana catesbeiana*, lead inhibits acquisition and retention of learning (Strickler-Shaw & Taylor 1991); tadpoles show a variety of sublethal responses such as changes in growth, development rate, pigmentation, the expression of morphological deformities, in less time than other biota, particularly humans (Berzins & Bundy 2002). The implications of reduced size, delayed development rate or malformations induced by lead in the environment may be severe for larvae. Indeed, a minimum body size must be reached before metamorphosis to give an adult amphibian (Wilbur & Collins 1973; Collins 1979) and to optimize chances of reproduction within the first year after metamorphosis (Smith 1987). Delayed metamorphosis and the lack of development attributed to lead exposure may influence the survival of larvae, since tadpoles which have a faster rate of growth may escape predation and decrease the risk of desiccation risk in ephemeral environments (Babbitt & Tanner 1997). Moreover, Lefcort *et al*. 1998 showed that exposure of *R. luteiventris* tadpoles to 5 and 50 mg Pb/l decreased the larval response of detection or reaction to predators. Consequently, exposure to lead in the environment, even at sub-lethal concentrations, seems to affect the general fitness for survival.

Usually, genotoxic effects are expressed at these sub-lethal concentrations of the tested substance. Indeed, genotoxic expression can be masked or limited by the expression of the acute toxicity. Formation of micronuclei is the consequence of chromosome fragmentation or malfunction of the mitotic apparatus and may result in genome mutations. In both cases, entire or fragmented chromosomes can no longer migrate to the cellular poles in the anaphase of the cell cycle resulting in a little clump of chromatin, called a micronucleus, near the principal nucleus in the cytoplasm of the daughter cell. Thus, clastogenic compounds and spindle poisons both lead to an increase in the number of micronucleated cells. Micronucleus induction is tributary to cellular division and hence to the mitotic index of the red blood cells.

In the present study, lead appeared to be genotoxic in erythrocytes of *Xenopus* larvae at 1, 10 and 30 mg/l. The clastogenicity of lead acetate has been shown *in vivo* with increased aberration frequencies in somatic and germ cells of mice (Aboul-Ela 2002) and in rat bone marrow cells (Tachi *et al*. 1985; Nehez *et al*. 2000). Lead also induces *in vivo* micronuclei in rat bone marrow cells (Nehez *et al*. 2000) and in lymphocytes of human workers (Vaglenov *et al*. 2001). Inorganic lead compounds are classified as a group 2B carcinogens (possible human carcinogen, IARC 1987)

and show clastogenic potential (DFG 2002). To our knowledge, no studies have been reported concerning the genotoxic effects of lead in amphibians. The present evaluation of lead genotoxicity in amphibian larvae is thus of essential ecotoxicological interest. Although biochemical and molecular mechanisms of lead toxicity are poorly understood, information is readily available about the indirect mechanisms of genotoxicity of lead (Hartwig *et al*. 1990; Valverde *et al*. 2001). Laterra *et al*. 1992 and Long *et al*. (1994) showed *in vitro* that lead effects may be due to its interference with calcium (Ca) in the activation of protein kinase C (PKC). Lead which may interfere with and /or mimic calcium in activating PKCs may act as a tumour promoter (Fracasso *et al*., 2002), due to interference with DNA repair processes, enhancing the genotoxicity of other damaging agents (UV, alkylating agents), and/or can take part in the Fenton reaction to generate hydroxyl radicals, singlet oxygen and other highly damaging reactive oxygen species (ROS) that are well known to cause DNA damage (Roy & Rossman 1992; Yang *et al*. 1999). Fracasso *et al*. (2002) showed that lead exposure induced comets owing to DNA damage in the lymphocytes of an occupationally exposed group, in correlation with high ROS production and low intracellular glutathione (GSH) levels. Thier *et al*. (2003) showed that formation of micronuclei was induced by inhibition of tubule formation and kinesin motility in V79 hamster fibroblasts treated with lead.

In the present study, the mitotic index in surviving larvae exposed to 10 mg Pb/l in experiment B and to 30 mg Pb/l in experiment A was reduced compared to the index in larvae from the other treatment conditions and the negative control. Surviving larvae showed morphological alterations in the great majority of erythrocytes ($>90\%$). Moreover, the blood samples did not contain enough cells to count. Consequently, MNE were not investigated in larvae exposed to 10 mg Pb/l in experiment B or to 30 mg Pb/l in experiment A, even though the number of individuals was statically satisfactory according to McGill's test criteria ($n > 7$, McGill *et al*. 1978). This is very likely related to the anaemia which became quickly established, even though erythrocyte destruction /removal and erythrocyte differentiation/multiplication occur simultaneously. Similar observations were reported in tadpoles where the number of red cells diminished after exposure to the metal (Barret 1947). Lead is well known to act on hematopoiesis in amphibians (Peri *et al*. 1998; Arrieta *et al*. 2000; Rosenberg *et al*. 1998, 2002) and elevated blood lead concentrations may be reflected in alterations of the morphology and the physiological properties of the circulating cells (Rosenberg *et al*. 2003). Lead is also known to alter red blood cell membrane proteins in humans (Caspers & Siegel 1980) and to produce reactive oxygen species involved in the phagocytic process as a consequence of altered membrane integrity, permeability and lipid peroxidation mechanisms (Gurer & Ercal 2000). Nevertheless, our observations were not reiterated in larvae exposed to 10 mg Pb/l in experiment A. In this case, it can be suspected that ions in the distilled water but originating from tap water may have limited the intoxication of larvae and consequently reduced the cytotoxicity of erythrocytes.

Accumulation of lead in liver was significant compared to the negative controls, for all concentrations. In amphibians, the liver is one of the primary target organs of metabolism, especially for metal accumulation (Vogiatzis & Loumbourdis 2001). In *Xenopus* larvae, the pathway of metal entry from exposure media is double: dermal (integument and gills) and breeding exposure. Indeed, most anuran larvae are detrituvorous (Bury & Whalen 1984), gill-breathing, microphageous feeders, thus leading to high ingestion rates of suspended particles (Wassersug 1975), especially particles of food susceptible to bind $Pb^{2+}$. Some investigations have proposed that Pb uptake of anuran larvae primarily occurs by ingestion of contaminated sediment (i.e., dietary intake) rather than from ingesting contaminated water (Jennet *et al*. 1977; Hall & Mulhern 1984; Birdsall *et al*. 1986; Sparling & Lowe 1996). Rice *et al*. (2001) proposed that Pb entered the body through ingestion rather than absorption across the tegument of the gills. Alvarado and Moody (1970) suggested that larval *R. catesbeiana* ingest water as a consequence of suspension feeding but they also appear to ingest water i.e. to "drink". Lead carbonate, an insoluble compound that is one of the major oxidation products of lead in water may be bio available to amphibians (Stansley *et al*. 1997). In the present study, lead in the liver increased as the amount of lead in the environment increased

and this is in agreement with of the report of Berzins and Bundy concerning whole *Xenopus laevis* exposed to lead from the embryonic to larval stages (Berzins & Bundy 2002). Chemical analysis performed on the positive control group of experiment A (which had no identified source of lead), showed that low contamination did occur during the exposure. Other, similar findings have been reported in whole body of control animals (Strickler-Shaw & Taylor 1991; Sobotka & Rahwan 1995). In our case, minimal contamination could come from the tap water, the diet source or from the CP added which might not have been completely exempt of metal traces.

In vertebrates, lead accumulates in bones and in kidneys, liver and gastrointestinal tract as well in red blood cells (WHO 1975) since lead cations are distributed throughout the soft tissues via the blood circulation. Subsequently, $Pb^{2+}$ will enter into the cell using the Ca-pump or Ca ion channels of the cell membranes, resulting in an efflux of Ca ions (Rosen & Pounds 1989; Simons 1988, 1993; Schanne *et al*. 1989). Consequently, it is not surprising that lead concentrations of 1, 10 and 30 mg Pb/l induce micronucleus formation in erythrocytes of *X. laevis* larvae in our study. Indeed, this result is closely related with accumulation of Pb in treated larvae. On the contrary, lower concentrations of $Pb^{2+}$ such as 0.1 and 0.01 mg/l did not induce genotoxicity in our experiment probably due to detoxification via metallothioneins for instance.

To sump up, lead is acutely toxic and genotoxic to amphibian larvae from 1 mg Pb/l and its bioaccumulation is significant from 1 $\mu$g Pb/l, potentially corresponding to environmental concentrations. Indeed, in the aquatic environment, Pb-concentrations are variable. Background concentration ranges from 0.003 $\mu$g/l in glaciers and 2 $\mu$g/l in lakes and rivers to 20 $\mu$g/l in groundwater and 36 $\mu$g/l in sediments interstitial waters (Nriagu 1978). Concentrations can range from 24 $\mu$g/l to 864 $\mu$g/l in draining waters from French dredged sediments, (Mouchet *et al*. 2005a) and from 98 $\mu$g/l (Barreaud et al. 1998) to 180 $\mu$g/l (Baladès *et al*. 1985) near French motorways in water streaming. However, more elevated concentrations have been reported for instance at shooting ranges (Stansley *et al*. 1992) and in eluates of contaminated soil ranging from 25 mg/l (Békaert *et al*. 2002) to 76 mg/l (Mouchet *et al*. 2006).

*Acute toxicity and genotoxicity of MBMCR alone*

MBMCR induced no signs of acute toxicity to *Xenopus* larvae. The growth of larvae exposed to MBMCR was even faster than that of the negative control group and no difference in larva size or food intake was noted, whatever the MBMCR concentration was. MBMCR, which rapidly settled to the bottom of the glass container, were ingested by larvae independently of the MBMCR mass in the water. Indeed, visual observation showed that inside the gut there were large white masses of ingested MBMCR starting from the lowest MBMCR concentration (0.1 g/l). MBMCR are rich in mineral salts directly available by larvae if ingested, and seem to be very nourishing.

MBMCR induced no micronucleus induction in *Xenopus* larvae. Moreover, micronucleus induction level in larvae exposed to MBMCR alone was significantly lower than those in negative control larvae, in the three experiments. This result indicates that MBMCR reduces the genetic damaging potential of elements present in the water used for the three experiments. The faster development larvae in the presence of MBMCR alone could also be due to the element-binding potential of MBMCR.

Concerning metal uptake, the present evaluation of MBMCR toxicity and genotoxicity is of great ecotoxicological interest. Moreover, it constitutes an innovative line of research since, to our knowledge, only one study has been published to this date, (Gulyurtlu *et al*. 2005). In the same way, these authors studying the eco-toxicity of ash resulting from MBM combustion with the Microtox assay (bacterial assay), also found no toxic effect of the ashes. In the present work, the evaluation of the toxicity-genotoxicity is carried out on aquatic amphibians, giving a major dimension to the environmental impact of MBMCR.

*Acute toxicity, genotoxicity and bioaccumulation of lead in the presence of MBMCR*

In the presence of MBMCR in water, no toxicity was noted in larvae at 0.1 and 1 mg/l Pb and less toxicity in larvae exposed to 30 mg/l. Moreover, Pb did not induce genotoxicity in *Xenopus* larvae exposed from 0.01 to 10 mg Pb/l, especially to 1 and 10 mg Pb/l, levels that were strongly genotoxic without MBMCR in water. In contrast, Pb

with MBMCR was genotoxic at 30 mg Pb/l which is not surprising considering the maximum binding capacity of MBMCR demonstrated previously by Deydier *et al.* (2003) during a chemical investigation. Indeed, the mechanisms and kinetics of lead removal by this bioinorganic natural apatite-rich material has already been investigated and compared to those of synthetic apatite (Deydier *et al.* 2003). Their results showed that MBMRC removes large quantities of lead (275 mg/g capacity, $< 300$ mg/g) in just a few minutes. This proportion of uptake does however remain lower than the theoretical maximum capacity (if Ca was totally substituted by lead). Stimulated by the ability of calcium hydroxyapatite (CaHA) to exchange cations (Suzuki *et al.* 1981) and by the low solubility of lead phosphates (Nriagu 1972, 1974), intensive research has attempted to account for the mechanisms involved in aqueous lead sequestration by synthetic apatites (Takeuchi & Arai 1990; Ma *et al.* 1994a, b; Valsami-Jones *et al.* 1998; Mavropoulos *et al.* 2002) and to evaluate the environmental applications. Immobilization of lead on synthetic or natural (bones, rocks...) CaHA is becoming a promising route for remediation of contaminated water (Takeuchi & Arai 1990; Ma *et al.* 1994a, b; Chen *et al.* 1997; Valsami-Jones *et al.* 1998; Admassu & Breese 1999; Mavropoulos *et al.* 2002).

Even though the presence of MBMCR prevented Pb from optimally reaching its cellular targets since the capacity of Pb-binding in the aqueous media probably partly accounts for the disappearance or the reduction of the toxicity and genotoxicity noted in larvae, accumulation of lead in liver in the presence of MBMCR was also significant compared to the negative control, irrespective of the lead concentration. This result suggests that even though Pb is physically bound to MBMCR in media exposure, lead uptake still occurred. In this case, lead particles mainly entered the organisms by ingestion of MBMCR by larvae feeding on the sediment at the bottom of the containers. This finding was confirmed by visual inspection of the feeding behaviour and of the gut content. Nevertheless, except for 0.01 mg/l Pb, accumulation of lead was lower in the presence than in the absence of MBMCR. Rice *et al.* 2001 demonstrated that food deprived frogs (*R. catesbeiana)* accumulated Pb to very high levels compared to larvae that were allowed to feed. They showed that this higher Pb-accumulation was not due to an increased uptake of Pb, but to a decreased Pb-elimination rate in unfed larvae. In the present case, it can be assumed that larvae exposed to MBMCR in water were fed more than larvae exposed without MBMCR, judging by the MBMCR content of their gut. Moreover, in agreement with MBMCR mineral composition, MBMCR seems to be nourishing since the growth of larvae exposed to MBMCR alone was greater that that of negative control larvae. Finally, lower levels of lead accumulation in larvae in the presence of MBMCR in water compared to those in the absence of MBMCR could also be related to higher defecating activity as observed daily in the glass containers. Most of the Pb from ingested MBMCR or water would be rapidly eliminated. The greater amount of Pb in larvae exposed to 0.01 mg/l in the presence of MBMCR, compared to the same concentration of Pb in the absence of MBMCR and to 0.1 and 1 mg Pb/l in the presence of MBMCR may be due to a differential capacity of elimination and/or to individual variability. Quarterman and Morisson (1975) established that the absorption and retention of lead in rats is especially influenced by Ca and phosphate which both decrease the retention and increase the excretion of lead. Meredith *et al.* (1977), Bogden *et al.* (1992) and more recently Varnai *et al.* (2001) also showed that higher Ca intake might be a way of reducing Pb absorption in rats. In the same way, Quaterman *et al.* (1978), Spickett and Bell (1983) and more recently Cortina-Ramirez *et al.* (2005) also showed that increased dietary phosphate limits Pb intestinal absorption in rats. It may be due to the Ca–Pb competition for the protein Ca transporters in the intestinal lumen (Edelstein *et al.* 1984; Fullmer 1997) and probably to formation of the phosphate–Pb complex, which makes the intestinal absorption of Pb difficult (Mikkanen *et al.* 1984). The evidence is that Ca-binding proteins have a high affinity for Pb (Fullmer *et al.* 1985; Simons 1986). Although sites of Ca flux have not been well investigated in larval stages of amphibians, these sites could be important in understanding Pb-accumulation because Pb and Ca are typically absorbed from and stored in the same tissue in humans (Barry 1975) and in fish (Hodson *et al.* 1984). In amphibians, some of the Ca stores that have been identified are endolymphatic sacs and skin (Stiffler 1996). Rice *et al.*

(2001) suggested that given that anuran larvae have poor assimilation abilities (Altig & McDearman 1975), it is possible that large amounts of Pb and Ca are ingested with water but little absorbed by the gut and hence made available for storage. Therefore, much of the Pb in the gut might have been contained just in the lumen and could well be unavailable for bioaccumulation. In our case, higher concentrations of ingested Ca in larvae could arise from ingested MBMCR which could contribute to reducing the availability of lead for bioaccumulation.

While lead uptake does indeed occur, the possible Pb complexes prevent the free circulation of lead and lower its bioavailability. Consequently, it is not surprising that concentrations of lead that were genotoxic in the absence of MBMCR in media exposure such as 1–10–30 mg Pb/l do not lead to micronucleus induction in erythrocytes of *X. laevis* larvae in the presence of MBMCR. *Xenopus* larvae seem to be more tolerant to Pb accumulation in the organism when Pb is immobilized on MBMCR.

## Conclusion

This study demonstrates that large concentrations of lead in water can be toxic to *Xenopus* larvae. Indeed, lead is acutely toxic and genotoxic to amphibian larvae from 1 mg Pb/l and its bioaccumulation is significant in the liver of larvae from the lowest concentration of exposure (0.001 mg Pb/l). Added to water, MBMCR were neither acutely toxic nor genotoxic in *Xenopus* larvae. MBMCR limits the expression of toxicity and genotoxicity of lead in *Xenopus laevis* larvae at concentrations that do not exceed the MBMCR capacity of Pb-binding. Pb accumulation in larvae exposed to lead with MBMCR in water was lower than Pb-accumulation in larvae exposed to lead alone except to 0.01 mg Pb/l suggesting complex mechanisms of MBMCR action in organisms. Our results suggest the possible use of MBMCR as an interesting element in the investigation of metal remediation. Heavy metals in aqueous solutions are usually removed by adsorption, ion exchange, coagulation, flotation, filtration, chemical precipitation, or reverse osmosis. Most of these methods require considerable cost. In contrast, MBMCR are low-cost materials since they are stockpiled in great amounts, especially in Europe. Indeed, since MBM were banned from animal feedstuffs in 2000 due to the risks of bovine spongiform encephalopathy, they have had to be incinerated. In this economical context, MBMCR could be technically analyzed in view of remediation studies and for their possible use as adsorbents, even if more mechanistic and environmental investigations are needed, especially in public health.

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