In vitro and *in vivo* studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetylcholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species

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The aim of this work was to assess the relationships between lead (Pb) and uranium (U) exposure, lipid peroxidation and some enzyme activities in a mollusc (*Corbicula* sp.), an earthworm (*Eisenia fetida*) and a fish (*Brachydanio rerio*). A comparative study was performed both *in vitro* and *in vivo* on whole organism postmitochondrial fractions and four potential biomarkers were analysed: a marker of neurotoxicity (acetylcholinesterase activity, AChE, EC 3.1.1.7), a marker of oxidative stress (malondialdehyde (MDA) level), and two markers of hydroperoxide detoxication: catalase (EC 1.11.1.6) and glutathione peroxidase (GPx, EC 1.11.1.9) activities. Our results have shown that the MDA contents were not significantly changed by exposures to lead either *in vitro* or *in vivo*. During uranium exposure, the MDA content was increased *in vitro* (particularly in fish samples) whereas this metal failed to stimulate lipid peroxidation *in vivo*. With some exceptions, *in vitro* and *in vivo* exposures to lead and uranium showed that the AChE, catalase and GPx activities were decreased in the three species. These exceptions indicated that different mechanisms occurred in the different species. In conclusion, it was shown that S9 fractions of whole organisms could be useful for environmental contamination biomonitoring. Moreover, it was shown that AChE activities were modulated by metals *in vivo* and cannot be considered as specific biomarkers of organophosphorus or carbamate pesticide exposure. Acetylcholinesterase and catalase activities could be used to survey lead and uranium contamination.

Keywords: *mollusc, fish, earthworm, lead, uranium, biomarkers.*

Introduction

Cellular injury from toxic metals may occur by a number of diverse mechanisms. It is generally established that metals affect the organism at a cellular level by (1) binding with soluble or membrane biomolecules such as enzymes, DNA or phospholipids, (2) reacting with SH group(s) of biomolecules such as glutathione, peptides or proteins and (3) affecting cellular metabolism such as transport across plasma membranes, mitochondrial function, lysosomal stability or DNA replication (Christie and Costa 1984, Goering *et al.* 1987, Viarengo *et al.* 1990). Apart from (1), all these could be caused by an alteration of the cellular membrane and numerous studies have shown that metal exposures enhance lipid peroxidation (LP). The effects of metals on LP processes have been largely established in mammals (humans, rats, rabbits, mice) either *in vitro* with exposures to iron, copper, cadmium, mercury, or vanadium or *in vivo* following exposures to iron, cadmium, mercury, vanadium, lead, cobalt, copper, nickel or tin (Ramstoeck *et al.* 1980, Christie and Costa 1984, Shafiq-ur-Rehman 1984, Sunderman 1985, Ali and Bondi 1989, Halliwell and Gutteridge 1990, Sugawara *et al.* 1991). It has also been shown, in fish, that LP is stimulated by various metals *in vitro* and/or *in vivo* (Radi and Matkovics 1988, Wofford and Thomas 1988, Bano and Hansan 1989, Winston and Di Giulio 1991, Thomas and Wofford 1993). Little is known about the effects of metals on LP in other species. Lawton and Donaldson (1991) have shown that dietary lead increased LP in chicken liver only for high doses (more than 1000 ppm). Viarengo *et al.* (1990), working on the gills and digestive gland of the mussel *Mytilus galloprovincialis*, demonstrated that copper induced LP whereas cadmium or zinc had no effect on this parameter. Moreover, recent evidence indicates that oxidative stress may occur in invertebrates (Di Giulio *et al.* 1989, Viarengo 1989, Livingstone *et al.* 1990, 1992, Ribera *et al.* 1990, 1991, Winston 1991, Winston and Di Giulio 1991).

The aim of this work was to assess the relationships between lead and uranium exposure, LP and some enzyme activities in a mollusc, an earthworm and a fish. These organisms have been chosen because they live in different biotopes and have different modes of nutrition (suspension feeder, detritivorous and carnivorous). Thus, they are representative of three parts of the ecosystem (soil, water and aquatic sediment) and could be used as sentinel organisms in the perspective of a global survey of the continental environment. Moreover, nothing or little is known about the effect of metals on their metabolism.

Earthworms are common in a wide range of soils. They have a number of characteristics which identify them as one of the most suitable soil animals for use as key bioindicator organisms for testing soils polluted by chemicals (Calahan 1988, Goats and Edward 1988). Therefore, they have been selected for ecotoxicological testing of industrial chemicals by the European Union (EEC 1984) and the Organization for Economic Co-operation and Development (OECD 1984). In this study, the species *Eisenia fetida andrei* was chosen. *Corbicula* sp. is a widely distributed freshwater bivalve. This species is generally recognized as an efficient bioaccumulator of contaminants (Graney *et al.* 1983, Hartley and Johnston 1983) and thus, it has been used by the US Environmental Protection Agency as a bioindicator of trace metals in fresh water (Elder and Collins 1991). The small teleostean fish

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Brachydanio rerio is widely used in laboratory experiments for the toxicological assessment of chemicals or industrial effluents. It is identified by the OECD guidelines as a test organism (OECD 1993a, b).

Lead (Pb) is a widely distributed metal that produces a spectrum of adverse effects in all animals. The natural lead content of fresh water has been estimated at about 1–10 $\mu g\ l^{-1}$. In soils, the average lead content is 16 $\mu g\ g^{-1}$. However, samples of top soils in urban areas have shown levels higher than 300 $\mu g\ g^{-1}$ (Beliles 1975). Investigations from a number of laboratories have demonstrated that lead enhances biological membrane alterations related to the formation of lipid peroxides (Lawton and Donaldson 1991).

Uranium (U) is a metal which is present in all soils and rocks in the form of a variety of minerals. The world average uranium content of soils is about 2 or 3 $\mu g\ g^{-1}$ soil. Uranium may occur in different oxidation states, the most stable and the most important, with regard to biological activity, is the hexavalent state. In hexavalent uranium compounds, which are easily soluble in water, uranium is found as the uranyl ion (UO_2^{2+}) (Wrenn *et al.* 1987, Bosshard *et al.* 1992). Because of its radiochemical properties and its interest in energy production, uranium has been largely studied to assess the safety of workers (linked to radiation). However, like all metals, uranium can induce a chemical toxicity but nothing or very little is known of the mechanism of this toxicity.

The objective of our work was to evaluate the effects of lead and uranium on potential biomarkers. Therefore, we performed a comparative study to identify the effects either directly on biomolecules (*in vitro* study) or after metabolic modulation (*in vivo* exposure). Moreover, these studies were performed on whole organism samples to highlight changes at individual levels. The results presented here concern a marker of neurotoxicity (the acetylcholinesterase activity, AChE, EC 3.1.1.7), a marker of oxidative stress (the malondialdehyde level (MDA), representing the state of membrane lipid peroxidation) and two markers of hydroperoxide detoxication: the catalase (EC 1.11.1.6) and the glutathione peroxidase (GPx, EC 1.11.1.9) activities.

MATERIALS AND METHODS

Test organisms

Worms were purchased from Ver'Humus (Cahors, France) and maintained in the laboratory in an artificial ground of reconstituted peat, as described by Roch (1980). Animals were kept in the dark at 21 °C. Mature worms were extracted from the culture media 24 h prior to exposure and stored on damp filter paper to void gut contents.

Clams of uniform size (2–2.5 cm shell length) were collected from the Sanguinet lake near Bordeaux (France). They were held in the laboratory for 4 days in tanks containing static aerated fresh water maintained at 20 °C. They were not fed during the acclimatizing period. Because clams are hermaphrodite (Kraemer *et al.* 1986), no sexual differences were taken into account.

Fish were purchased from Le Lann (Gradignan, France). Animals of uniform size (3–3.5 cm length) were held in the laboratory for 7 days as described for clams. However, they were fed during the acclimatizing period using commercial food for fish (TetraMin from Tetrawerke, Melle, Germany). No sexual determination was made before sampling.

Study designs

In vitro exposure

Pools of 50 fish, clams or worms were prepared in order to obtain enough material of exactly the same sample to carry out all the *in vitro* studies. For each biochemical measurement, small aliquots of metal solutions (from 0 to 5 mg metal per litre in a maximum volume of 30 µl) were added to the assay mixture (see Analytical Procedures) in order not to significantly change the mixture volume. To determine the longest time of contact between metals and sample, incubations of different durations were tested (5, 10, 15, 20 and 30 min). It appeared that 5, 10 or 15 min did not significantly change the catalase, AChE or glutathione per-oxidase activities respectively. Thus, these optimal durations of contact were chosen for each measurement. The temperatures of incubation were identical to those used for each biochemical measurement. All measurements were made in triplicate.

In vivo exposure

Animals were kept for 11 days as described in Table 1. They were not fed during the testing period. Twenty animals were used per dose. At the end of the testing period, for each dose, animals were divided into three pools, each pool containing six or seven individuals. Each measurement was carried out on each pool of animals.

Preparation of samples

All procedures were carried out at 4 °C. Whole fish, whole worm and soft tissues of molluscs were homogenized in Tris buffer (100 mM, pH 7.5) in a 1/3 w/v (weight/volume) ratio for 1 min with a T25 Ultra Turrax at 9000 rpm. The homogenate was then centrifuged for 30 min at 9000 *g* in a Sigma 3MK centrifuge. Aliquots of the supernatant (S9 fraction) were frozen at –80 °C until use.

Chemicals

Lead acetate, uranyl acetate, glutathione (GSH), glutathione reductase (GR, EC 1.6.4.2), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), hydrogen peroxide (H_2O_2), butylated hydroxytoluene (BHT) and cumene hydroperoxide (CUOOH) were purchased from Sigma-Aldrich Chimie S.A.R.L. (Saint Quentin-Fallavier, France). Reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) was from Boehringer Mannheim (Meylan, France). Other chemicals were of 'rigorously pure' grade.

Analytical procedures

The variation of optical density (OD) was quantified by a thermoregulated Perkin Elmer 550S differential spectrophotometer.

Total protein contents were determined according to the method of Lowry *et al.* (1951), adapted to the Technicon Autoanalyser by Shuel and Shuel (1961).

Acetylcholinesterase activities were measured according to methods adapted from Ellman *et al.* (1961). Acetylthiocholine (AThCh) was hydrolysed by acetylcholinesterase, producing thiocholine and acetic acid. The released thiocholine reacts with 5,5′-dithio-bis-2-nitrobenzoate (DTNB) to produce 5-thio-2-nitro-benzoate (TNB), a yellow compound which absorbs at 412 nm (absorbance 13.6 $mM^{-1}\ cm^{-1}$).

The reaction conditions were 87 mM Tris, pH 7.5, 0.35 mM DTNB, 1.96 mM AThCh, 0.2 mg sample proteins per ml incubate. The reaction was conducted at 30 °C and initiated by addition of AThCh.

Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents according to the method described in Livingstone *et al.* (1990). Tissues were homogenized in 21% trichloracetic acid containing 1 mM BHT and centrifuged at 5000 *g*. The supernatant was then mixed with 0.75% thiobarbituric acid and heated at 100 °C for 15 min. Following further addition of 70%

Organisms	System	Exposure conditions	Tested concentrations of metals
Aquatic			
Brachydanio rerio	Aerated static fresh water system	20 animals/dose 5 fish per litre	From 0 to 1090 mg Pb per litre From 0 to 750 µg U per litre
Corbicula sp.		20 animals/dose 10 clams per litre	From 0 to 250 mg Pb per litre From 0 to 200 mg U per litre
Terrestrial *Eisenia fetida*	Dishes with moistened filter paper daily aerated as described in OECD (1984)	20 animals/dose 1 worm per dish with 2 ml metal solution	From 0 to 314.36 µg Pb or U per cm^2 filter paper

Table 1. *In vivo* experimental design.

AChE activities (nmol min^{-1} mg^{-1} protein)					
Metal concentration (mg Pb per litre)	0.00	2.50	25.00	250.00	2500.00
Eisenia fetida andrei	65.91±1.44	60.67±2.77**	38.57±2.99**	24.11±2.64****	22.90±3.94****
Corbicula sp.	3.24±0.43	2.76±0.33	2.21±0.15****	2.21±0.42***	1.66±0.19****
Brachydanio rerio	59.13±0.60	58.82±2.56	57.77±0.84***	57.42±2.35	38.52±2.55****

MDA levels (nmol mg^{-1} protein)								
Metal concentration (mg Pb per litre)	0	10	50	500	818	1000	1500	3000
Eisenia fetida andrei	0.31±0.12	0.23±0.05	0.29±0.12	0.29±0.02	0.36±0.02	0.15±0.02*	0.34±0.00	0.19±0.19
Corbicula sp.	0.37±0.07	0.31±0.05	0.32±0.05	0.21±0.10	nm	0.42±0.35	0.54±0.08	0.49±0.06
Brachydanio rerio	0.60±0.25	0.61±0.41	0.53±0.00	0.31±0.02	nm	0.48±0.13	0.35±0.08	0.70±0.25

Catalase activities (nmol min^{-1} mg^{-1} protein)						
Metal concentration (mg Pb per litre)	0.00	137.50	275.00	412.50	682.00	2500.00
Eisenia fetida andrei	30.30±0.38	33.84±0.87***	29.55±0.76	25.76±1.52***	22.73±1.52***	22.32±0.38****
Corbicula sp.	8.08±0.87	13.26±0.38****	11.36±0.76***	11.62±0.87***	18.94±0.76****	18.69±0.44****
Brachydanio rerio	15.53±0.38	15.15±0.00	13.26±0.38***	11.74±0.38****	11.74±0.38****	11.68±0.29****

GPx activities (nmol min^{-1} mg^{-1} protein)				
Metal concentration (mg Pb per litre)	0.00	500	1500	5000
Eisenia fetida andrei	4.32±0.75	4.15±0.36	4.41±0.21	3.26±0.14
Corbicula sp.	7.91±1.19	5.70±0.55*	5.46±0.54*	3.48±0.58***
Brachydanio rerio	7.05±0.88	4.30±0.61**	4.24±0.80**	3.22±0.18***

Table 2. *In vitro* effects of lead on biochemical measurements.

Lead concentrations are expressed as mg l^{-1} in the reaction mixture. AChE: Acetylcholinesterase; MDA: malondialdehyde; GPx: glutathione peroxidase. Results are expressed as mean±SD (n = three pools of seven animals for each measurement). Means were compared by F- and t-tests for independent samples using the Statistica 4.5 software (Statsoft Inc. Ed.).

Key: * = $p < 5\%$; ** = $p < 2\%$; *** = $p < 1\%$; **** = $p < 0.1\%$; nm, not measured.

trichloracetic acid, the MDA-like peroxide products were quantified by reference to MDA (absorbance 156 mM^{-1} cm^{-1}). The MDA-like lipid peroxides are the TBARS present in the tissue at the time of sampling and are an indicator of the degree of *in vivo* lipid peroxidation. This method is limited by the fact that other reactive aldehydes may not be detected and that the MDA produced *in vivo* may react with other cellular constituents (Halliwell and Gutteridge 1985).

Catalase activities were assayed as described in Greenwald (1985): the variations of absorbance (at 240 nm) caused by the dismutation of hydrogen peroxide were measured (absorbance 40 M^{-1} cm^{-1}, reaction conditions: 63 mM Tris, pH 7.0, 50 mM H_2O_2, 3 µg sample proteins per ml incubate, temperature of incubation 20 °C).

The glutathione peroxidase activities were monitored by following the decrease of NADPH (at 340 nm) consumed during reformation of reduced glutathione (GSH) from oxidized glutathione (GSSG) according to Greenwald (1985) (absorbance 6.2 mM^{-1} cm^{-1}, reaction conditions: 75 mM Tris, pH 7.4, 1.5 mM

AChE activities (nmol min^{-1} mg^{-1} protein)					
Metal concentration (mg U per litre)	0.00	2.50	25.00	250.00	2500.00
Eisenia fetida andrei	65.91±1.44	56.25±3.56****	57.46±1.63****	51.43±3.15****	56.25±2.11****
Corbicula sp.	3.24±0.27	3.87±0.13****	3.32±0.28	2.76±0.14***	2.77±0.20***
Brachydanio rerio	59.13±0.60	58.82±1.71	50.77±0.46****	54.27±0.32****	54.62±0.67****

MDA levels (nmol mg^{-1} protein)								
Metal concentration (mg U per litre)	0.00	0.56	1.12	2.80	56.00	560.00	1120.00	2800.00
Eisenia fetida andrei	0.66±0.06	0.54±0.10	nm	0.61±0.07	0.79±0.35	0.82±0.23	0.72±0.04	0.76±0.01*
Corbicula sp.	0.75±0.13	0.61±0.08	nm	0.71±0.06	0.86±0.29	0.81±0.23	0.87±0.12	1.43±0.02****
Brachydanio rerio	1.15±0.15	1.47±0.03**	1.88±0.69*	nm	1.91±0.23****	1.85±0.12****	2.31±0.43****	2.32±0.06****

Catalase activities (nmol min^{-1} mg^{-1} protein)						
Metal concentration (mg U per litre)	0.00	137.50	275.00	412.50	682.00	2500.00
Eisenia fetida andrei	30.30±0.38	30.81±0.58	26.26±0.58****	25.00±0.76****	22.35±0.38****	22.10±0.22****
Corbicula sp.	8.08±0.87	14.77±0.38****	13.26±0.38****	15.53±0.38****	15.91±0.76****	15.40±0.44****
Brachydanio rerio	15.53±0.38	12.12±0.76	10.99±0.38****	10.99±0.66****	10.61±0.38****	10.29±0.29****

GPx activities (nmol min^{-1} mg^{-1} protein)				
Metal concentration (mg U per litre)	0	500	1500	5000
Eisenia fetida andrei	4.32±0.75	4.32±0.64	5.18±0.47	5.90±0.44
Corbicula sp.	5.83±0.28	5.48±1.60	6.05±0.33	6.83±0.41*
Brachydanio rerio	7.05±0.88	4.66±0.48**	5.18±0.97	6.62±0.47

Table 3. *In vitro* effects of uranium on biochemical measurements.

Uranium concentrations are expressed as mg l^{-1} in the reaction mixture. AChE: Acetylcholinesterase; MDA: malondialdehyde; GPx: glutathione peroxidase. Results are expressed as mean±SD (n = three pools of seven animals for each measurement). Means were compared by F- and t-tests for independent samples using the Statistica 4.5 software (Statsoft Inc. Ed.)

Key: * = $p < 5\%$; ** = $p < 2\%$; *** = $p < 1\%$; **** = $p < 0.1\%$); nm: not measured.

GSH, 120 µM NADPH, 25 mM CUOOH for *Corbicula* and *Eisenia* measurements or 20 mM for *Brachydanio* measurements, 3 Units GR for *Corbicula* measurements or 2 Units GR for *Brachydanio* and *Eisenia* measurements, 12 µg *Corbicula* proteins per ml incubate or 10 µg *Brachydanio* or *Eisenia* proteins per ml incubate). The reaction was conducted at 25 °C and initiated by addition of CUOOH. As observed in Livingstone *et al.* (1992), the GPx activities are changed by freezing the samples. Thus, in order to compare samples, the GPx activities were measured in samples frozen for precisely 24 h.

Statistical procedures

Means were compared by F- and t-tests for independent samples using the Statistica software (release 4.5A, Statsoft Inc. Ed., 1993).

Results

In vitro study

The results are presented in Table 2 (lead) and Table 3 (uranium).

Significant decreases of the AChE activities were observed in the subcellular fraction of the three species studied, both with lead and uranium. Lead appeared to be the most important inhibitor of the AChE activities (the maximum observed decreases were up to 65% in worms, 49% in clams and 35% in fishes for Pb doses of 2.5 g l^{-1}). These maximum decreases were less important for uranium exposures (30% in worms and 15% in clams or fishes for U doses of 0.25 g l^{-1}).

The MDA levels were increased in the S9 fraction of *Eisenia fetida*, *Brachydanio rerio* and *Corbicula* sp. exposed to uranium. Only the highest dose of uranium (2.8 g l^{-1}) induced statistically significant changes in *Eisenia* and *Corbicula*. In *Brachydanio*, a significant increase (28%) was observed from the lowest dose (0.56 mg l^{-1}). This increase reached a maximum of 101% for the highest doses (2.8 g l^{-1}). Apart from one measurement, no statistically significant result was observed with lead exposures. Changes owing to lead contamination were variable and no trends were highlighted.

The catalase activities showed a consistent decrease in the S9 fractions of *Eisenia fetida* and *Brachydanio rerio*. The maximum decreases were of the same order (about 30%) in both species for the two metal exposures. The effects observed in *Corbicula* were the opposite: the catalase activities were strongly increased with lead and uranium addition. These changes reached 134% and 97% respectively for lead and uranium doses of 682 mg l^{-1}.

	Metal concentrations	AChE activities (nmol min^{-1} mg^{-1} protein)	MDA levels (nmol mg^{-1} protein)	Catalase activities (nmol min^{-1} mg^{-1} protein)	GPx activities (nmol min^{-1} mg^{-1} protein)
Eisenia fetida andrei exposures (µg Pb per cm^2)	0.0	61.05±6.29	0.67±0.08	15.80±1.48	3.09±0.37
	0.5	37.49±4.76****	0.82±0.03**	10.73±0.74****	3.09±0.36
	1.0	21.01±0.93****	0.48±0.05***	11.87±1.24***	2.94±0.30
	5.0	22.99±3.51****	0.27±0.03****	15.15±2.14	3.25±0.09
	10.0	28.84±2.88****	0.24±0.04****	9.66±0.89****	2.98±0.40
Corbicula sp. exposures (mg Pb per litre)	0	2.28±0.34	0.80±0.13	6.23±1.18	4.20±0.44
	10	1.33±0.21	1.11±0.04***	3.54±0.44**	4.06±0.04
	50	1.66±0.77	0.77±0.05	3.28±0.44**	3.57±0.06*
	100	1.54±0.85	0.37±0.05****	3.30±0.00***	4.03±0.70
	250	2.31±0.50	0.30±0.03****	2.02±0.44***	4.24±0.28
Brachydanio rerio exposures (mg Pb per litre)	0	59.76±5.62	0.89±0.060	10.29±2.70	6.58±1.47
	10	33.91±5.04****	0.65±0.020****	18.94±1.89***	5.36±0.61
	20	25.15±4.36****	0.43±0.030****	11.11±0.88	5.02±0.18
	40	27.74±2.74****	0.54±0.015****	9.85±2.00	5.00±0.42
	100	16.04±3.68****	0.60±0.042****	3.03±0.38***	4.41±0.36*

Table 4. Biochemical measurement variations after *in vivo* exposures with lead.

AChE: Acetylcholinesterase; MDA: malondialdehyde; GPx: glutathione peroxidase. Results are expressed as mean±SD (n = three pools of seven animals for each measurement). Means were compared by F- and t-tests for independent samples using the Statistica 4.5 software (Statsoft Inc. Ed.).
Key: * = $p < 5\%$; ** = $p < 2\%$; *** = $p < 1\%$; **** = $p < 0.1\%$.

	Metal concentrations	AChE activities (nmol min^{-1} mg^{-1} protein)	MDA levels (nmol mg^{-1} protein)	Catalase activities (nmol min^{-1} mg^{-1} protein)	GPx activities (nmol min^{-1} mg^{-1} protein)
Eisenia fetida andrei exposures (µg U per cm^2)	0.0	61.05±6.29	0.67±0.08	15.80±1.48	3.09±0.37
	0.5	41.52±1.93***	0.83±0.07**	14.55±1.73	3.16±0.06
	1.0	45.30±2.37***	0.49±0.05***	12.88±1.36***	3.12±0.04
	5.0	35.31±12.99***	0.38±0.03****	19.19±6.19	3.51±0.11
	10.0	27.63±5.91****	0.36±0.03****	10.61±1.36****	3.31±0.39
Corbicula sp. exposures (mg U per litre)	0	2.28±0.34	0.80±0.13	6.23±1.18	4.20±0.44
	10	1.65±0.58	0.80±0.02	2.90±0.58***	4.13±0.26
	50	1.38±0.48*	0.47±0.15**	2.46±1.15***	4.38±0.19
	100	1.22±0.00**	0.45±0.02***	2.27±0.76***	4.58±0.25
	200	1.36±0.24***	0.46±0.01***	2.53±0.44***	4.02±0.09
Brachydanio rerio exposures (mg U per litre)	0.000	59.76±5.62	0.89±0.060	10.29±2.70	6.58±1.47
	0.075	48.76±2.05**	0.47±0.031****	8.71±1.52	6.40±0.80
	0.150	45.32±11.81*	0.34±0.041****	7.32±2.18	7.85±0.49
	0.300	33.96±6.81****	0.30±0.041****	8.21±2.46	6.91±1.06
	0.750	31.73±3.17****	0.28±0.020****	8.21±0.88	5.61±0.62

Table 5. Biochemical measurement variations after *in vivo* exposures with uranium.

AChE: Acetylcholinesterase; MDA: malondialdehyde; GPx: glutathione peroxidase. Results are expressed as mean±SD (n = three pools of seven animals for each measurement). Means were compared by F- and t-tests for independent samples using the Statistica 4.5 software (Statsoft Inc. Ed).
Key: * = $p < 5\%$; ** = $p < 2\%$; *** = $p < 1\%$; **** = $p < 0.1\%$.

Surprisingly, the lowest tested dose (U or Pb doses of 137.5 mg l^{-1}) produced a greater increase in this activity when compared with the effects of doses of (275 mg l^{-1}). GPx activities, measured after lead exposure, were decreased both in fish and clams. These decreases were dose-related and reached 56% and 54% in, respectively, *Corbicula* and *Brachydanio* for the highest dose tested (5 g l^{-1}). When exposed to uranium, this activity was also inhibited in these animals. However, the lowest activities were observed for the first dose tested (0.5 g l^{-1}) and addition of greater amounts of uranium produced smaller decreases. In earthworms, lead seemed to inhibit GPx activities (not significant 25% decrease for the highest dose of 5 g l^{-1}), whereas uranium produced an increase of the activity (up to 36% for 5 g l^{-1} but not statistically significant).

In vivo study

The results are presented in Table 4 (lead) and Table 5 (uranium).

The AChE activities were generally decreased by exposure to lead and uranium. This decrease was observed for every species exposed to uranium, and reached 55% for *Eisenia*

(at 10 mg cm^{-2}) and 47% for *Corbicula* (at 100 mg l^{-1}) and *Brachydanio* (at 0.75 mg l^{-1}). For this last species, the decrease was dose dependent. The inhibitions as a result of lead exposure were also strong in worms (up to 66% at 1 μg cm^{-2}) and fish (up to 73% at 100 mg l^{-1}). Whereas the three lowest doses of lead induced a decrease of the AChE activities in clams, in comparison with the control, we observed no changes for the highest dose (25 mg l^{-1}).

All the MDA levels declined in *Brachydanio* exposed to lead and uranium. This decrease reached 69% at uranium doses of 0.75 mg l^{-1} and 52% at lead doses of 20 mg l^{-1}. In *Corbicula* and *Eisenia*, except at the lowest doses where they were superior or equal to the control, the MDA contents were decreased by exposures to lead and uranium. This decrease was higher for lead exposure (worms: 64% at 10 μg cm^{-2} and clams: 63% at 250 mg l^{-1}) than for uranium exposure (up to 46% in worms and 44% in clams).

In *Corbicula* the catalase activities were highly decreased by exposure to lead and uranium. This inhibition was at least 43% and 53% for 10 mg l^{-1} of lead and uranium respectively. Moreover, it was dose-related to lead exposure. The catalase activities of *Eisenia* were lowered by exposure to metals (Pb and U). This decrease was very low and not significant for lead doses of 5 μg cm^{-2}. Moreover, at the same dose of uranium, we also observed a non-significant increase (21%) of this activity. In *Brachydanio*, catalase activities were slightly decreased by uranium exposure. In contrast, lead exposure produced different changes: for low doses, the activity was increased (84% at 10 mg l^{-1}), whereas it was decreased for the high doses (70% at 100 mg l^{-1}).

Slight variations of GPx activities occurred in the three species. No significant changes were observed in *Brachydanio* and *Corbicula* exposed to uranium and in *Eisenia* exposed to both uranium and lead. For *Corbicula*, this activitiy was significantly decreased (15%) by exposure to lead doses of 50 mg l^{-1}. Fish (*Brachydanio*) GPx activities were inhibited (33%) by the highest concentration of lead (100 mg l^{-1}).

Discussion

The significant decreases in AChE activities observed *in vitro* in the three species, either with lead or uranium, points to the inhibiting effect of these metals. Our results are in accordance with those of Galzigna *et al.* (1969), Heywood *et al.* (1978), and Olson and Christensen (1980) who observed, *in vitro*, a strong decrease of the AChE activities in mammal samples exposed to triethyl or tetraethyl lead (more than 70% of inhibition) or fish samples exposure to pure chemicals.

This inhibiting power, confirmed by our *in vivo* study, suggests a direct effect of metal on the AChE enzymes.

Some authors assume that AChE activities are exclusively indicators of organo-phosphorus or carbamate pesticides exposure (Hill and Fleming 1982, Galgani and Bocquene 1989, Day and Scott 1990, Bocquene and Galgani 1991). In this study we have shown that they are also modulated by metals *in vivo*. Thus, AChE activities must not be considered as specific biomarkers. Moreover, the inhibitions observed during field studies must be interpreted as both pesticide and/or metal exposure.

Numerous metals are able to induce LP *in vitro* and/or *in vivo*. In the three species, our *in vitro* studies have shown that exposures to lead produced no changes in the MDA contents but that they were enhanced by the exposures to uranium. Moreover, the MDA measurements were lowered by *in vivo* exposures to lead or uranium for three of the four tested doses.

Thus, it seems that lead did not induce reactive oxygen species (ROS) generation either *in vitro* or *in vivo*. During uranium exposures, results were more conflicting: the metal failed to stimulate LP *in vivo* whereas it enhanced the MDA contents *in vitro* (particularly in fish samples). Such differences between *in vivo* and *in vitro* exposures have been previously reported by Thomas and Wofford (1993) with cadmium. Moreover, in accordance with these authors, we cannot conclude because of the enhancement of the MDA content alone, that reactive oxygen species were produced during our experiments and that such (ROS-mediated mechanisms are the basis of the toxicity of lead or uranium for the clam, the worm or the fish.

Antioxidant enzymes such as catalase and GPx play a major role in protecting cells from oxidative damage: catalase metabolizes H_2O_2 to H_2O and O_2 whereas GPx metabolizes either H_2O_2 or organic hydroperoxides (preventing either the ROS toxicity or the chain propagation reactions involved in LP). These enzymes are inducible under conditions of oxidative stress (Di Giulio *et al.* 1989). However their activities are regulated by a number of other conditions including changing nutritional status or oxygen tension (Livingstone *et al.* 1992).

Various trends were observed in the MDA content *in vivo*: it was increased for low doses in *Eisenia fetida* (for Pb and U exposures) and *Corbicula* sp. (for Pb exposures) whereas higher doses produced a significant decrease. Identical changes between low and high doses were observed for catalase activities in *Brachydanio rerio* (for Pb exposures). These trends have been previously described for *in vivo* exposure to cadmium and mercury in fish erythrocytes (Gwozdzinski *et al.* 1992), by a mix of metals (Cu, As, Cd, Sn, Hg and Pb) in marine and freshwater molluscs (Rodriguez-Ariza *et al.* 1992) and by organic oxiradical generators in marine mussels (Livingstone *et al.* 1990, Ribera *et al.* 1991).

As previously described by Posthuma and Van Straalen (1993), these variations could also be due to adaptive mechanisms in relation to long term exposures and high metal concentrations. The occurrence of adaptive mechanisms could also explain the surprising decrease in MDA content observed *in vivo*. Indeed, these results could be caused either by a stabilization of the membranes (and thus a lower sensitivity to metal-induced damage) or by increased metabolism of lipid peroxides.

In vitro exposures to lead and uranium, showed that (1) AChE activities were decreased in the three species, (2) catalase activities were inhibited in worms and fish and (3) GPx activities were lowered in all organisms except in worms exposed to lead. Similar decreases of the AChE, catalase and GPx activities were observed *in vivo* (with some exceptions as previously described).

These results confirm, both *in vitro* and *in vivo*, the enzyme-inactivating power of lead and uranium. It is generally recognized that this inactivation is linked to the properties of

metals to bind with sulphydryl hydroxyl, carbonyl, imidazole and amino residues of proteins, peptides, amino acids and the –NH and –C=O groups of the protein chain backbone (Viarengo 1989).

The strong increase in the catalase activities observed *in vitro* in clams exposed to lead or uranium may evoke another mechanism of toxicity. This activity was assayed in the S9 fraction where the activity comes from broken peroxisomes and cytosolic catalase. Moreover, the activity in the 9000 *g* pellet (because of the intact peroxisome) was not quantified. Thus, if peroxisomes remained in clams S9 fraction, the effect of metals on the leakage of enzyme from peroxisomes and not a direct effect on catalase enzymes was observed and measured.

In conclusion, it has been shown that S9 fractions of whole organisms could be useful for environmental contamination biomonitoring because some biochemical responses were strongly affected by exposures to lead or uranium, both *in vitro* and *in vivo*. However, their usefulness is somewhat limited for studying mechanistic aspects of xenobiotic toxicity like explaining the *in vivo* decrease of MDA content.

In this context, AChE and catalase activities seem to be useful for the biomonitoring of lead and uranium contamination. Future research needs to be conducted in field conditions to confirm these results.

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