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Genotoxic and stress inductive potential of cadmium in *Xenopus laevis* larvae

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Abstract

The present investigation evaluates the toxic potential of Cd in larvae of the frog *Xenopus laevis* after 12 days of exposure to environmentally relevant contamination levels, close to those measured in the river Lot (France). Several genotoxic and detoxification mechanisms were analyzed in the larvae: clastogenic and/or aneugenic effects in the circulating blood by micronucleus (MN) induction, metallothionein (MT) production in whole larvae, gene analyses and Cd content in the liver and also in the whole larvae.

The results show: (i) micronucleus induction at environmental levels of Cd contamination (2, 10, 30 $\mu g\,L^{-1}$); (ii) an increased and concentration-dependent quantity of MT in the whole organism after contamination with 10 and 30 $\mu g\,Cd\,L^{-1}$ (a three- and six-fold increase, respectively) although no significant difference was observed after contamination with 2 $\mu g\,Cd\,L^{-1}$; (iii) Cd uptake by the whole organism and by the liver as a response to Cd exposure conditions; (4) up-regulation of the genes involved in detoxification processes and response to oxidative stress, while genes involved in DNA repair and apoptosis were repressed.

The results confirm the relevance of the amphibian model and highlight the complementarity between a marker of genotoxicity, MT production, bioaccumulation and genetic analysis in the evaluation of the ecotoxicological impact.
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1. Introduction

Increased environmental pollution can be attributed to a variety of factors resulting from new industrial and agricultural technologies, together with changes in our way of life. In addition, the nature of the pollution itself has become more diverse. Metallic pollution receives widespread attention because of increasing amounts of metals being released into the environment in relation with the development of industrial societies. Regardless of its origin, pollution tends to find its way into the aquatic environment.

In freshwater, metal, such as cadmium, concentration ranges from 2 to 30 $\mu g\,L^{-1}$ (Audry et al., 2004). Cadmium is expected to be persistent in ecosystems and toxic to a wide variety of organisms. It has no biological role and is not an essential metal. It exerts a large number of adverse effects on ecosystems and human and animal health (IPCS, 1992). Among them, genotoxic effects may durably affect the aquatic ecosystems and their presence in water can also have repercussions on non-aquatic species, via food chains, or simply as a result of drinking the water.

A number of tests have been developed to assess the genotoxic potency of water samples, using either plants or aquatic animals (see the reviews of Jaylet et al., 1990; Godet et al., 1993). The assays can be carried out with intact animals, taking into account uptake and elimination, internal transport and metabolism. An example is the use of amphibians, which have proved to be valuable bioindicators and a sensitive model for environmental studies (AFNOR, 2000; Jaylet et al., 1986; Krauter et al., 1987; Gauthier, 1996; Ferrier et al., 1998; Zoll et al., 1990; Djomo et

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al., 2000; Mouchet et al., 2005a, 2006). Larvae can be reared, not only in containers filled with unconcentrated water samples (laboratory conditions) but also in running water of various sources (factory effluents, surface water, river waters, etc.). 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, clastogenic compounds and spindle poisons both lead to an increase in the number of micronucleated cells. Induction of micronuclei has been widely used for genotoxicity testing in amphibians (Jaylet et al., 1986; Krauter et al., 1987; Mouchet et al., 2005b).

In particular, the ecotoxicological relevance of the amphibian *Xenopus laevis* has already been well established in a large array of studies focused on biomarkers such as the induction of biotransformation enzyme activities (Békaert et al., 2002; Gauthier et al., 2004), micronucleus formation (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; Bonfanti et al., 2004).

Heavy metals can enter cells in different ways, such as membrane transport of ions or passive diffusion of neutral chemical species (Delnomdedieu et al., 1992). In cells, they can increase reactive oxygen species (ROS) formation and thus generate oxidative stress (Stohs and Bagchi, 1995) leading to lipid peroxidation in mitochondria and *in fine* to the release of cytochrome *C*, which constitutes one of the last steps of cell apoptosis (Konigsberg et al., 2001). Oxidative stress can also generate DNA damage leading again to apoptotic or necrotic cell death. To fight against oxidative stress, cells possess anti-oxidant defences involving enzymes like catalase, superoxide dismutase (SOD) or glutathione peroxidase (Stohs and Bagchi, 1995). Different mechanisms could be involved in metal detoxication, for instance, detoxication dependent on low molecular weight cysteine-rich proteins called metallothioneins (MT). These proteins are able to chelate and sequestrate seven metal ions (zinc or cadmium) per MT molecule in mammals (Chan et al., 2002). However, chelation capacities differ between metals and depend on the chemical forms in which the metal is present (Vasak and Hasler, 2000). The production of this kind of protein is known to be stimulated in the event of Cd exposure in aquatic organisms (Legeay et al., 2005; Gonzalez et al., in press). Another mechanism of detoxication is based on the active efflux of metals via pumps belonging to the family of ATP-binding-cassette (ABC) transporters. These are membrane proteins that use ATP as an energy source and are able to extrude both xenobiotics and metals actively (Dassa and Bouige, 2001; Achard et al., 2004).

In order to evaluate the genetic effects of Cd contamination in *X. laevis* larvae, 15 genes were selected which encode for proteins involved in anti-oxidant defences, metal chelation, active efflux of xenobiotics, mitochondrial metabolism, DNA repair and apoptosis. Three genes are known to be involved in the oxidative stress response: cytoplasmic and mitochondrial superoxide dismutases (*sod* and *sodMt*) and the heat shock protein *hsp70* genes. The mitochondrial metabolism was investigated using the pyruvate carboxylase (*pyc*) and the cytochrome *C* oxidase subunit I (*coxI*) genes. Two other genes are putatively involved in detoxication mechanisms: the MT gene reported for *X. laevis* (*mt1*) and a gene belonging to the ABC transporter family (*tap*). The *tap* gene was chosen for its homologies with multidrug-resistance transporters like the human *MDR1* (Gottesman and Pastan, 1993), the *leishmania LtpgpA* (Dey et al., 1996) or the yeast *YCF1* (Li et al., 1997) genes. Four genes involved in Cd-induced apoptotic mechanisms were selected: *c-jun*, *c-fos*, *p53* and *bax* (Lag et al., 2002). Three genes are known to be expressed in DNA damage repair: the growth-arrest-DNA-damage (*gadd*), the *mutL* and the *rad51* genes (Chen and Shi, 2002). The *actin* gene, due to its constitutive expression, was used as a reference gene.

The aim of the present work is to evaluate the induction of micronuclei, metallothioneins and genes in response to cadmium stress at relevant environmental contamination levels with regards to cadmium content in *Xenopus* larvae. Cd concentrations for *Xenopus* exposure were based on environmental concentrations found in the river Lot, near the Decazeville region (Aveyron, South-West of France). To our knowledge, no studies concerning the induction of micronuclei, metallothioneins and genes in response to metallic stress such as cadmium in amphibians have been reported.

2. Materials and methods

2.1. Xenopus, breeding and maintenance

Eggs, fertilized in vitro, from sexually mature *X. laevis* were provided by the National Breeding Department of Rennes-1, University (Rennes, France). A female was injected with 700 IU of human chorionic gonadotropin (HCG) to induce spawning. Eggs were obtained by back massage and covered with a suspension of sperm. In vitro fertilization of *X. laevis* eggs was performed and maintained in Marc's modified Ringers solution (MMR) medium (0.5 mM HEPES, pH 7.8, 10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.01 mM EDTA). The larvae were then maintained in chlorine-free water at $22 \pm 0.5\,^{\circ}C$ until they reached a developmental stage appropriate for experimentation.

2.2. Exposure conditions

Exposure began with larvae at stage 50, according to the *Xenopus* table of development (Nieuwkoop and Faber, 1956). The larvae were taken from the same laying to reduce inter-animal variability. Groups of 40 larvae (100 mL/larva) were exposed in 4-L polyethylene tanks containing the control medium (negative and positive) or the test medium (various concentrations of $CdCl_2$). The negative control was chlorine-free water supplemented by nutritive salts (294 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 123.25 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 64.75 mg L^{-1} $NaHCO_3$, 5.75 mg L^{-1} KCl, pH 7). A positive control was performed to check the responsiveness of the amphibian larvae using addition of cyclophosphamide (CP [6055-19-2], Sigma, France) at 20 mg L^{-1} (dissolved in water) to water. The concentrations of Cd^{2+} tested in mineral water were: 2, 10, 30 μg L^{-1}.

Cadmium was applied as $CdCl_2$ (Merck, France). Throughout the experiment, the larvae were subjected to a 12-h light:12-h dark cycle and were fed every day with dehydrated aquarium fish food. The tanks were partially immersed in water baths to maintain the temperature at $22 \pm 0.5\,^{\circ}C$. The media in the control and experimental tanks were permanently oxygenated. They were renewed daily to eliminate larval faeces and the remains of food. Contamination of the water was based on daily additions of aqueous solutions of Cd. The amounts of Cd added were adapted to compensate for the decrease in metal concentrations over the 24-h cycles. The decrease in Cd concentration was measured on a regular basis by analyzing water samples (10 mL). At the end of exposure, all the larvae reached stage 54 at about the same time, according to the *Xenopus* table of development and were immobilized by immersion in tricainemethane sulfonate (MS222, Sandoz). The genotoxic and detoxification biomarkers (micronuclei and metallothioneins), the Cd bioaccumulation and the genetic analyses were performed in larvae which did not show any signs of acute toxicity after visual inspection (such as death, reduced size, corporal malformations, diminished food intake or abnormal behavior). Analyses of micronuclei and bioaccumulation of Cd were performed on 20 larvae on a random basis per condition. Analysis of metallothioneins and of genes was performed on 20 other larvae.

2.3. Micronucleus test (MNT) procedure

The *Xenopus* MNT was carried out according to the Standard AFNOR NF T90-325 procedure (AFNOR, 2000). After 12 days of exposure, a blood sample was obtained from each immobilized larva by cardiac puncture. After fixing in methanol and staining with hematoxylin, the smears were screened under a 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 for each condition.

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 and the medians and quartiles calculated. The statistical method used to compare the medians was based on the recommendations of McGill et al. (1978) 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. 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.

2.4. Cd analysis

Dissected tissues (liver and whole organisms) were thawed, dried on absorbent paper and weighed (wet weight). Samples were digested by nitric acid (3 mL of pure HNO_3) under pressure in borosilicate glass tubes at 100 °C for 3 h. After dilution of the digestates to 20 mL with ultrapure water (MilliQ plus), cadmium concentrations were measured by atomic absorption spectrophotometry with Zeeman correction, using a graphite tube atomizer (AAS, Thermoptec M6 Solaar). In order to avoid interference, analyses were carried out in the tube atomizer with a blend of Pd and $Mg(NO_3)_2$. The detection limit was 0.1 $\mu g\,Cd\,L^{-1}$ (3× standard deviation of the reagent blanks). The analytical method was simultaneously validated for each sample series by the analysis of standard biological reference materials (TORT-2, lobster hepatopancreas; DOLT-2, dogfish liver; NCR/CRNC, Ottawa, Canada). Values were in agreement with the certified ranges (data not shown). The results are expressed in average metal concentrations accumulated in organs (three replicates per organ and per exposure condition) in nmol $Cd\,g^{-1} \pm$ standard error (S.E.), wet weight.

2.5. Metallothionein quantification

The levels of total MT proteins in the entire larvae were determined by the mercury-saturation assay, as previously described, using cold inorganic mercury (Dutton et al., 1993; Baudrimont et al., 2003). Analysis of MTs was conducted in three replicates (100 mg of tissue from two pooled *X. laevis*) per exposure condition, the saturation assay being repeated twice per sample. This technique is based on the quantification of Hg bound to the saturated MTs. The denaturation of non-MT proteins was performed with trichloroacetic acid and the excess Hg not bound to the MTs was removed with lyophilised beef hemoglobin (Sigma) prepared in 30 mM Tris–HCl buffer (pH 8.2 at 20 °C). The final supernatant was then quantitatively recovered and used for Hg determination by flameless atomic absorption spectrometry (AMA 254, Altec, Prague, Czech Republic). The detection limit was estimated at 1 ng Hg. Owing to the fact that the exact quantity of Hg binding sites per MT molecule is unknown for this species, MT concentrations cannot be directly expressed in nmol $MT\,g^{-1}$ (wet weight), but in nmol $Hg\,g^{-1}$ (wet weight).

2.6. Total RNA purification

Total RNAs were extracted from 40 mg of fresh tissue on three replicates (pools of two larvae) using the Absolutely RNA RT-PCR Miniprep kit (Stratagene), according to the manufacturer's instructions. The quality of all RNAs produced was evaluated by electrophoresis on a 1% agarose–formaldehyde gel, and their concentration determined by spectrophotometry. For each exposure condition, sample analyses were carried out in triplicate.

2.7. Reverse transcription of RNA

First-strand cDNA was synthesized from 5 μg total RNA using the Stratascript First-Strand Synthesis System (Stratagene). Briefly, RNAs were adjusted to 38 μL with DEPC-treated water. After addition of 2 μL of oligo(dT) and 1 μL of random primers, the reaction was incubated at 65 °C for 5 min. Then, 40 units of Rnase inhibitor, 2 μL of 100 mM dNTPs, 5 μL of 10× first-strand buffer and 50 units of Stratascript reverse

Table 1
Accession numbers and specific primer pairs for the 16 *X. laevis* genes used in our study

Gene name	Accession number	Primer (5′–3′)
mt1	M96729	CTGAAGGGCTCTACGTCTGC[a] CCCCAAATGTCACCCAAAGT[b]
tap	AY204551	TTGCTGTCGGTGGGGC[a] CAGTGTTGATTCGGTGGG[b]
coxI	M10217	CCGGAGGAGGTGACCC[a] CTCAGACAATAAAGCCTAGAATCCA[b]
hsp70	BI939172	CCCAGCGACAGGCTAC[a] AGTGTTCACCAGCGGTTAGA[b]
pyc	BC059308	ACAGATCAGGACACAGGCA[a] CCTAGTCCCATACTGTGGGC[b]
sod	X51518	GTGTGCTGGCGGGAAG[a] CCGGGGCTCCGTGATT[b]
sodMt	AY362041	TGTCAGTTGGAGTCCAGGG[a] AGTTGATAACATTCCAGATCGCTTT[b]
actin	BE490926	ATTGAGCCACCAATCCAGG[a] ACAAGTGTTGGAATGTGCG[b]
globin	J00978	CTGAGAGTGGACCCTGGC[a] GTTGAGACTTCTGCTGCTGT[b]
c-jun	AJ243954	TGTGTGCGGGAAGTCCT[a] ACCTAGTGTCTCTAGCAGTTTAACC[b]
c-fos	AJ224511	CTCATGCCGGTGCTGG[a] CTGGGTATGTGAAGACAAATGATGTT[b]
p53	M36962	GGTTCGAGTGTGTGCCTG[a] CGCCCCTTAATCCGCAA[b]
bax	AY437085	AAGCAAGGGGAAGGCG[a] CGGGTCACTCTGTTGGG[b]
gadd	BC044046	AAGACAATCAGGATGTCGCT[a] CCTTGATCTGTAATTCATGTGGGT[b]
rad51	BC046650	ATTCCTATGCAATGGCCGT[a] GGCGCTTGCCTCCAGTAA[b]
mutL	BE681219	TATGAGGACTGAGAAAACAGAAGCA[a] AGTTACAAACACTTGGAGCTGAT[b]

Abbreviations: *mt*, metallothionein; *coxI*, cytochrome *C* oxidase subunit I; *pyc*, pyruvate carboxylase; *sod*, cytoplasmic superoxide dismutase; *sodMt*, mitochondrial superoxide dismutase; *hsp*, heat shock protein; *gadd*, growth-arrest-DNA-damage.
[a] Upstream primer.
[b] Forward primer.

transcriptase were added. The reaction was incubated for 1 h at 42 °C in an Eppendorf Mastercycler. The cDNA mixture was conserved at −20 °C until its use in real-time PCR reaction.

2.8. Primer design

The accession numbers of the 16 genes used in our study are reported in Table 1. For each gene, specific primer pairs were determined using the LightCycler probe design software (version 1.0, Roche). All the primer pairs are reported in Table 1.

2.9. Real-time PCR

Real-time PCR reactions were performed in a LightCycler (Roche) following the manufacturer's instructions (1 cycle at 95 °C for 10 min, and 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s). Each 20 μL reaction contained 2 μL of reverse transcribed product template, 1 μL of master mix including the SyberGreen I fluorescent dye (Roche), enabling the monitoring of the PCR amplification, and the gene-specific primer pair at a final concentration of 300 nM for each primer.

Reaction specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SyberGreen fluorescence level during gradual heating of the PCR products from 60 to 95 °C. The relative quantification of each gene expression level was normalized according to the actin gene expression. In this way, for each gene expression level, the mean value and the associated standard deviation ($n = 3$) were determined. The mean values for each exposure level were statistically compared to those of the control condition using the non-parametric Mann–Whitney *U*-test. From this comparison, induction factors of each gene were obtained by comparing each mean value observed in the contaminated water with that of the control water. Then, only the statistically significant expression level changes ($p < 0.05$) were used.

3. Results

3.1. Micronucleus test

The micronucleus test results are presented in Fig. 1. The median value of micronucleated erythrocytes per thousand, MNE‰ for the negative control was 0 ± 0.35. The positive control showed a significantly higher MNE‰ compared to the negative one. MNT results reveal that cadmium was genotoxic to amphibian larvae at all the concentrations tested. Indeed, the

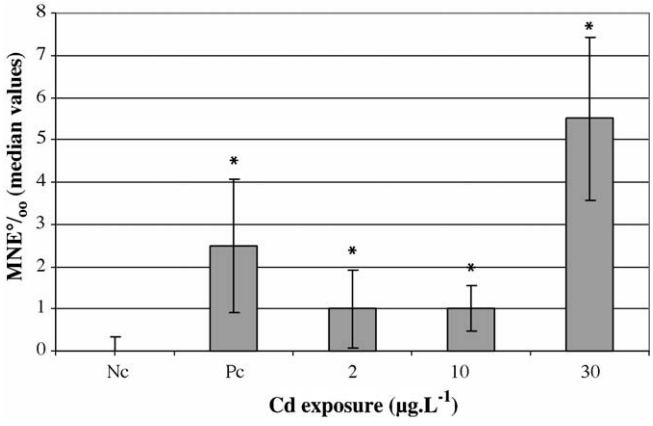

Fig. 1. Results of the *X. laevis* micronucleus test. Level of micronucleated erythrocytes (MNE‰) after 12 days of Cd-treatment. The micronucleus test was carried out with 20 larvae per concentration exposed for 12 days to 2, 10 and 30 μg L^{-1} of Cd. The vertical bars show the inter-quartile ranges. Asterisks indicate a genotoxic result (McGill et al., 1978). Nc: negative control (nutritive water); Pc: positive control (20 mg L^{-1} CP).

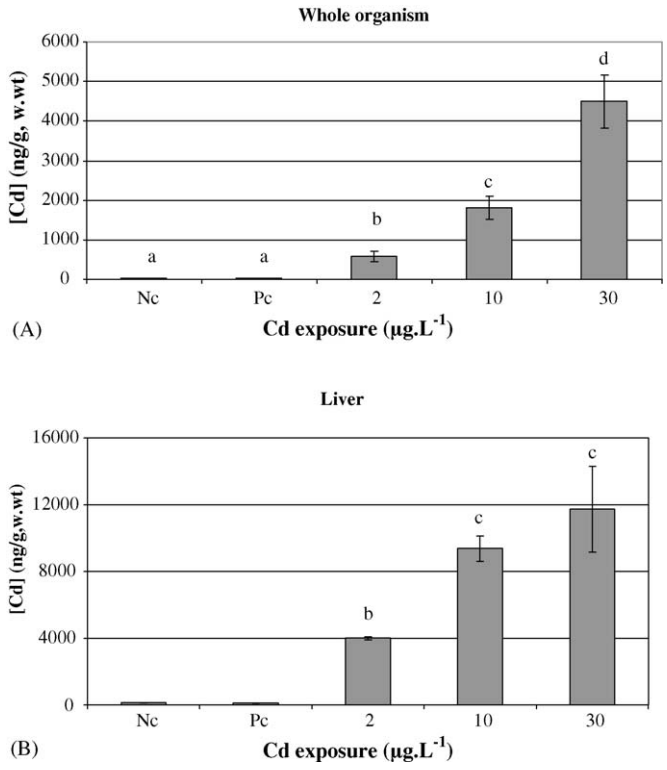

Fig. 2. Mean cadmium concentrations (ng Cd g^{-1} tissue, wet weight ± S.E.M., $n = 3$) determined in tissues from whole *X. laevis* larvae (A) and in liver (B) after 12 days of Cd exposure. Nc: negative control (nutritive water); Pc: positive control (20 mg L^{-1} CP). The different letters correspond to significant differences between conditions of exposure ($p < 0.05$, Mann–Whitney *U*-test).

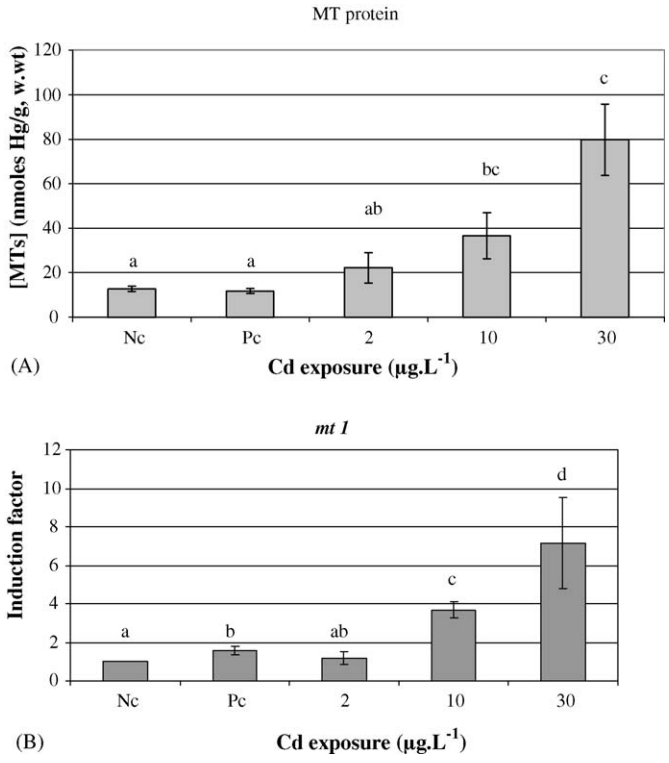

Fig. 3. Quantification of MT proteins (A) and induction of *mt1* expression levels (B) determined in whole *X. laevis* larvae (mean ± S.E.M., $n = 3$). Nc: negative control (nutritive water); Pc: positive control (20 mg L^{-1} CP). The different letters correspond to significant differences between conditions of exposure ($p < 0.05$, Mann–Whitney *U*-test).

results were significant at a median value of 1 for a 95% confidence limit for 2 and 10 µg Cd L^{-1}. At the higher concentration (30 µg Cd L^{-1}), the median value of MNE‰ increased to a value of 5.5‰. Nevertheless, no clear dose–response relationship was apparent.

3.2. Cd bioaccumulation in tissues of X. laevis

Bioaccumulation of Cd was determined at the whole organism level and in liver. Results showed the same tendency for the two kinds of samples (Fig. 2). Indeed, only little Cd was detected in positive and negative controls, while a dose-dependent bioaccumulation was observed for Cd-treated individuals. At the whole organism level, accumulation of Cd ranged from 572.8 ± 122 to 4498.7 ± 665 ng Cd g^{-1} wet weight for larvae receiving from 2 to 30 µg Cd L^{-1}, respectively. The same tendency was observed when bioaccumulation of Cd was determined in liver. Indeed, metal accumulation increased according to Cd exposure conditions and ranged from 4000 ± 76 to 11,730.8 ± 2566 ng Cd g^{-1} wet weight for larvae receiving from 2 to 30 µg Cd L^{-1}, respectively.

3.3. Metallothionein concentrations and gene expression

No significant differences were observed, during MT protein quantification, between controls and larvae treated with cyclophosphamide or 2 µg Cd L^{-1} on the level of the whole organism (Fig. 3). In contrast, a concentration-dependent increase of MT was noted after contamination with 10 and 30 µg Cd L^{-1}. Indeed, a three- and six-fold increase was determined for these two conditions, respectively, corresponding to 36 and 79 nmoles Hg g^{-1} (wet weight). The same tendency was observed when the genetic expression level of *mt*1 was investigated. Indeed, compared to control basal expression, no differences existed after contamination with 2 µg Cd L^{-1}, while four- and seven-fold increases were noted after treatment with 10 and 30 µg Cd L^{-1}, respectively. However, in contrast to protein quantification, MT mRNAs were significantly more abundant (1.6-fold increase) in individuals treated with cyclophosphamide than in their control counterparts.

3.4. Gene expression levels

Gene expression levels were investigated by quantitative real-time RT-PCR in liver and in the remaining tissues. The results indicated tissue-specific basal levels of expression (Table 2). Indeed, genes involved in the response to oxidative stress (cytoplasmic and mitochondrial *sod*), mitochondrial metabolism (*cox*I) and detoxification (*mt1*) were more abundantly expressed at the basal level than the actin gene when the whole organism was considered. The same was true when the basal expression levels of genes were investigated in liver. When the basal expression of the two tissues was compared, all the investigated genes were expressed more in the liver than in the other larval

Table 2
Comparative basal expression for the selected genes observed in liver, and in whole *X. laevis* larvae

Function	Genes	Liver	Whole organism	Liver/WO
Mitochondrial metabolism	*coxI*	128	4	32
	pyc	1	0.25	4
Oxidative stress	*sod* (*Cu/Zn*)	64	16	4
	sod (*Mn*)	8	4	2
	hsp70	0.5	0.125	2
	globin	256	0.125	2048
Detoxification processes	*mt1*	64	8	8
	tap	0.5	0.06	8
DNA repair	*gadd*	1	0.125	8
	rad51	1	0.125	8
	mutl	2	0.125	16
Apoptosis	*p53*	2	1	2
	bax	1	0.25	4
	c-jun	0.25	0.25	1
	c-fos	0.125	0.125	1

The actin gene was used as the reference gene.

tissues. For example, *globin* and *mt1* were 2048- and 8-fold more expressed in the liver than on the scale of the whole organism.

Compared to the control organisms, gene expression, in the whole positive control organism showed up-regulation of *pyc*, *cox*I, *mt*1, *globin* and *sodMt* genes, while the *c-fos* gene levels decreased (Fig. 4A). In the same way, numerous genes were differentially regulated in contaminated larvae. The apoptotic *bax* gene was repressed in every cadmium exposure. In contrast, *cox*I and *sodMt* genes were up-regulated during treatment with 2 and 10 μg Cd L^{-1}, but returned to basal level after the exposure to the highest Cd concentration. The heat shock gene *hsp70* increased during contamination with 2 μg Cd L^{-1}, but was clearly repressed for the two remaining treatments. Decreased expression levels were observed for the *globin* and the *c-fos* genes after contamination with 10 and 30 μg Cd L^{-1}, while their expression remained at basal levels in the lowest exposure condition. Surprisingly, the *tap* gene was only repressed during exposure to 10 μg Cd L^{-1}.

In the liver, the same trend as above was observed concerning the *c-fos* (decrease) and *sodMt* (increase) genes. However, marked differences existed for the remaining genes (Fig. 4B). Indeed, like in the whole organism, *mt1* gene expression was up-regulated in liver, although the up-regulation seemed smaller than that for whole organism. In contrast, *cox*I, *hsp70* and *tap* genes were repressed at the three Cd levels. A clearly increased expression of *pyc* appeared after treatment with 10 and 30 μg Cd L^{-1}. Surprisingly, as was observed for the positive control, expression of the genes involved in DNA repair mechanisms (*rad51*, *gadd* and *mutL*) were down-regulated after contamination with 30 μg Cd L^{-1}, concomitantly with the expression of the apoptotic *bax* gene. However, all these genes were up-regulated after exposure to 10 μg Cd L^{-1}.

4. Discussion

The micronucleus test revealed a genotoxic response after exposure of *Xenopus* larvae to relatively low concentrations of Cd (2, 10 and 30 μg L^{-1}). The main originality of our study was the use of environmentally realistic exposure conditions as encountered in the river Lot (Audry et al., 2004). Indeed, it should be stressed that in most studies dealing with various animal models and cell cultures, the authors used extremely high Cd

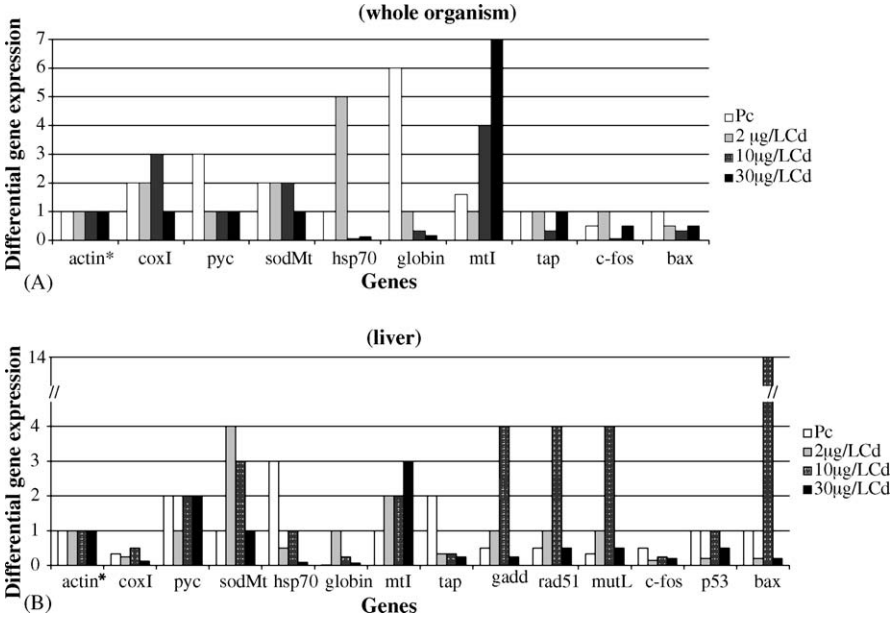

Fig. 4. Differential gene expression observed (A) in whole larvae and (B) in liver compared to controls from *X. laevis* after 12 days of contamination with cyclophosphamide (Pc) and three Cd concentrations (2, 10 and 30 μg L^{-1}). Results are given as induction (>1) or repression (<1) factors as compared to control *X. laevis*. Only genes presenting statistically significant expression level changes ($^{*}p < 0.05$) were indicated. The actin gene was used as the reference gene.

levels, bearing no relation to environmental pollution. In a previous study, micronucleus induction was also obtained after 12 days of exposure of *X. laevis* larvae to 2 mg Cd L^{-1} ($CdCl_2$) but not with 0.25, 0.5 or 1 mg Cd L^{-1} and no micronucleus induction was noted after 12 days of exposure of *Pleurodeles waltl* larvae to 2, 1, 0.5, 0.25 mg Cd L^{-1}. Although cadmium is suspected to have a spindle activity and is known for its likely carcinogenic, mutagenic and teratogenic activity (I.A.R.C., 1987, 1993a,b), cytogenetic bioassay data are conflicting (Hartwig, 1994; Forni, 1992; Verougstraete et al., 2002) probably because of the indirect effects of Cd. Indeed, even if the mechanisms underlying the toxic effects of cadmium are still not well understood, Cd is known to induce DNA strand breaks and chromosomal aberrations but its mutagenic potential is rather weak (Hartwig, 1994; Waisberg et al., 2003). Cadmium is also known to induce genotoxicity *via* oxidative stress (Filipic and Hei, 2004; Devi et al., 2001), DNA binding (Hassoun and Stohs, 1996), or inhibition of DNA repair activities (Lützen et al., 2004; Hartwig, 1998). Consequently, it is not surprising that low concentrations such as those used in the present work, can lead to micronucleus induction in *X. laevis* larvae.

This result is closely related with the accumulation of Cd in treated larvae. In *Xenopus* larvae, cadmium can enter the system in two ways: dermal (skin and gills) and breeding exposure. Indeed, most anuran larvae are gill-breathing, microphageous feeders, thus leading to high ingestion rates of suspended particles (Wassersug, 1975), especially particles of food which could probably bind Cd^{2+}. In mammals, the liver is one of the primary target organs of metabolism, especially for metal accumulation. To our knowledge, published studies concerning cadmium uptake in *X. laevis* exclusively used to earlier stages of embryonic development (Herkovits et al., 1998) such as embryos up to stage 47 (Nieuwkoop and Faber, 1956). In *Xenopus leavis* embryos, based on lethality data, susceptibility to cadmium seems to increase gradually from the two-blastomere stage up to stage 40 while an increase in resistance occurs at stage 47 (Herkovits et al., 1997). Nebeker et al. (1995) reported 13.7 mg L^{-1} wet weight Cd in the larvae of the salamander *Ambystoma gracile* exposed for 24 days to 504.5 ppb Cd. Feeding the same animal with food containing 1173 mg L^{-1}, resulted in a concentration of about 50 mg L^{-1} wet weight Cd, which corresponds to 400–450 mg L^{-1} dry weight. Experiments with adults of *Rana ridibunda* exposed to 200 mg L^{-1} for 30 days, recorded about 900 mg L^{-1} dry weight (Vogiatzis and Loumbourdis, 1998).

Such a high Cd content probably leads to MT induction. Indeed, from the first day of exposure the larvae could establish an effective detoxification mechanism, theoretically capable of "minimizing" the toxic effects of cadmium. A well studied and effective mechanism of detoxification is the production of metallothioneins, which are able to bind seven zinc or cadmium metal ions in mammals (Goyer, 1992; Chenon et al., 2002). Numerous studies have been carried out on the use of MT as biomarkers in relation to the contamination of aquatic species by heavy metals (Couillard and Saint-Cyr, 1997). Metallothioneins are known to be involved in the detoxification of toxic metals like cadmium (Hamer, 1986) and play a predominant role in Cd depuration (Baudrimont et al., 2003). The sensitivity of cells or tissues to cadmium appears to be related, at least in part, to MTs biosynthesis and previously to expression of the *MT* gene. Activation of the *MT* gene can limit the genotoxic effects of cadmium. Cadmium ions are very efficient inducers of MT mRNA accumulation in *X. laevis* liver (Muller et al., 1993). Nevertheless, MT induction was probably insufficient to inhibit the clastogenic effects of cadmium and finally the micronucleus induction in our exposure conditions (daily renewal of the Cd contamination). Could spillover support this situation? "Spillover" corresponds to the metals still remaining due to the overwhelmed defense capacities of the larvae when confronted with a massive uptake of metals in cells and tissues, resulting in rapid and severe structural and functional damage. This theory was first described by Winge et al. (1974) who stipulated that once the MTs in the cytosol are saturated by metals and the intracellular fluxes of toxic elements are beyond their biosynthesis capacities, sequestration by MTs and thus protection are overwhelmed and the metals exert their toxicity. This has been clearly described in aquatic organisms such as the bivalve mollusc *C. fluminea* (Baudrimont et al., 1999). This process would lead to stabilization of MT levels over the range of the Cd concentrations applied. In contrast, we observed a positive and significant correlation between Cd bioaccumulation, metallothionein concentrations ($R = 0.8$, $p < 0.01$, Spearman correlation) and micronucleus induction. On the other hand, it should be noted that for 2 μg Cd L^{-1}, despite a significant Cd bioaccumulation, no MT production was observed to parallel MN induction. This means that the functional pool of MT is sufficient to scavenge cytosolic Cd but also that Cd is able to exert deleterious effects on DNA via different pathways. Moreover, some other mechanisms could be involved in Cd detoxification in order to inhibit micronucleus induction. In this way, a recent study of *C. fluminea* reported that xenobiotics like Cd could be extruded from the cell by active efflux after forming a complex with glutathion (Achard et al., 2004; Achard-Joris et al., 2006). From our results, it can be concluded that this mechanism is not efficient in *X. laevis* (no induction of the *tap* gene).

The genetic analysis revealed significant but relatively low overexpression of the *mt1* gene under cyclophosphamide (CP) exposure, while no differences were observed when protein levels were determined. CP is widely used as an alkylating agent in tumour chemotherapy (Chabner and Collins, 1990). The results of Wei et al. (1999) show that metallothionein can covalently sequester the active form of cyclophosphamide in vitro. From these results, discrepancies between MT gene and protein levels observed during our experiment could be explained. MT proteins are quantified by the Hg saturation assay based upon the binding of Hg to MT. The uptake of CP by MT could decrease the access of Hg to binding sites in the MT clusters. Consequently, an underestimation of MT protein levels could occur using this method. This result demonstrated that the MT gene and protein levels give complementary information and should be analyzed together. The overexpression of the *mt1* gene under CP exposure also indicated that CP is able to induce the expression of MT.

No difference was observed between the MT gene and protein inductions under Cd contamination, leading to a strong

correlation between these two parameters ($R = 0.7$, $p < 0.05$, Spearman correlation). This indicated that MTs in *X. laevis* are only regulated at the transcriptional and not at the translational level. This suggests that *X. laevis* larvae possess a high metabolic activity and that probably all transcribed genes are translated into protein. This fact could be in relation with the high levels of metabolic activity occurring in the premetamorphic stages of these organisms.

Overexpression at the whole organism level was greater than in the liver. However, it should be noted that basal expression of most of the genes investigated was higher in the liver than in the whole organism. In this context, basal expression appeared sufficient to fight against low contamination pressure in the whole organism since no significant difference between control and $2\,\mu g\,L^{-1}$ contaminated larvae was detected. The same results were recently observed in zebrafish, where the liver exhibited the highest level of expression for numerous genes (Gonzalez et al., in press). This high level of expression in the liver compared to other organs was correlated to the high metabolic activity of this tissue which constituted one of the primary targets of metals during exposure.

Genes encoding for anti-oxidant enzymes like *sodMt* and *coxI* were induced in larvae, indicating that contamination by Cd generated oxidative stress in the cells. Cadmium is known to accumulate in mitochondria and is able to disrupt the mitochondrial transport chain, leading to the formation of free radicals and hence lipid peroxidation (Stohs and Bagchi, 1995; Company et al., 2004). Surprisingly, expression of *sodMt* and *coxI* in the whole organism returned to basal levels during exposure to $30\,\mu g\,L^{-1}$. The same holds true for the expression of *sodMt* in liver, while liver *coxI* remained repressed whatever the exposure conditions. These results suggest that the tissues brought other molecular mechanisms into action to fight against Cd at the highest exposure concentration.

Genetic analysis in liver revealed the down-regulation of genes involved in DNA repair after exposure to $30\,\mu g\,Cd\,L^{-1}$, while expression of these genes was up-regulated at $10\,\mu g\,L^{-1}$ and remained unchanged compared to controls at $2\,\mu g\,L^{-1}$. This suggested that the DNA damage generated by Cd could be repaired when exposure concentrations were low. On the contrary, Cd seems to inhibit DNA repair mechanisms at high exposure concentration. In this way, Cd is known to generate DNA damage, such as DNA breaks, *via* the production of oxygen-reactive species (Waisberg et al., 2003). Our results, in *X. laevis*, are in agreement with a recent study reporting the inhibiting effect of Cd on human DNA repair mechanisms in vivo (Lützen et al., 2004). Indeed, Cd has been shown to inhibit the DNA–protein interactions essential for the initiation of nucleotide excision repair, but also to inhibit the activity of replication protein A, a protein required for complete mismatch repair. Such processes could be hypothesized in *X. laevis* after exposure to high doses of Cd. It should be noted that genetic analysis revealed that the down-regulation of DNA repair genes was concomitant with the down-regulation of apoptotic genes. Classically, cells with damaged DNA are expected to arrest at the G2 checkpoint, and if damage is not repaired, to initiate apoptosis (Cejka et al., 2003). From these results, the down-regulation of apoptotic genes in *X. laevis* could be an indirect consequence of the inhibition of DNA repair mechanisms by Cd, since the cells could not be stopped in G2.

5. Conclusion

These results focused on the combined analysis of metallothionein and micronucleus induction, gene analysis and Cd content after exposure of *X. laevis* larvae at environmentally realistic levels of Cd contamination. Since the micronucleus test, metallothionein levels, bioaccumulation and gene analyses provide complementary information, their use on the same biological model is recommended. In spite of the low levels of Cd (corresponding to the environmental concentrations encountered in the Lot river), genotoxic effects were observed in *X. laevis* larvae, thereby confirming the ecotoxicological impact of supposedly tolerable background levels.

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