Comparative Evaluation of the Toxicity and Genotoxicity of Cadmium in Amphibian Larvae (*Xenopus laevis* and *Pleurodeles waltl*) Using the Comet Assay and the Micronucleus Test

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ABSTRACT: The toxic and genotoxic potential of Cadmium ($CdCl_2$) were evaluated by the micronucleus test (MNT) and comet assay (CA) using amphibian larvae (*Xenopus laevis* and *Pleurodeles waltl*). Acute toxicity results showed that Cd is toxic to *Xenopus* larvae exposed from 2 to 50 mg/L and to *Pleurodeles* from 5 to 50 mg/L, depending on the nature of the water (reconstituted water containing mineral salts or mineral water MW (Volvic®)). The MNT results obtained in MW showed that Cd (2 mg/L) is genotoxic to *Xenopus*, whereas it was not genotoxic to *Pleurodeles* at all concentrations tested. The CA established that the genotoxicity of Cd to *Xenopus* and *Pleurodeles* larvae depends on the concentration, the exposure times, and the comet parameters (Tail DNA, ETM, OTM, and TL). The CA and MNT results were compared for their ability to detect genotoxic effects, considering the concentrations of Cd applied and the exposure time. The CA showed Cd to be genotoxic from the first day of exposure. In amphibians, the CA appears to be a sensitive and suitable method for detecting genotoxicity such as that caused by Cd.
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Keywords: cadmium; amphibian; *Xenopus laevis*; *Pleurodeles waltl*; acute toxicity; genotoxicity; comet assay; micronucleus test

INTRODUCTION

Increased environmental pollution can be attributed to a variety of factors resulting from current 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 metal being released into the environment due to the development of industrial societies. This is an area of increasing concern in industrialized countries. Regardless of their origin, metals tend to find their way into the aquatic compartment and can exhibit toxic and genotoxic activity in fresh water.

Cadmium is used in metal plating, metallurgical alloying, mining, ceramics and pigments and in other industrial

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techniques such as the production of chemical stabilizers (Martins et al., 2004) as well as in agriculture as a by-product (Coles et al., 1995). It 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 activities may durably affect the aquatic ecosystems and cadmium's presence in water can also have repercussions on nonaquatic species, via food chains, or simply as a result the water being used for drinking.

A number of tests have been developed, using either plants or aquatic animals (see the reviews of Jaylet and Zoll, 1990; Godet et al., 1993), which can potentially serve to assess the genotoxic potency of pollutants in water samples. 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 (Jaylet et al., 1986; Krauter et al., 1987; Zoll et al., 1990; Gauthier, 1996; Ferrier et al., 1998; AFNOR, 2000; Djomo et al., 2000; Mouchet et al., 2005a, 2006a). Larvae can be reared, not only in containers filled with unconcentrated water samples (laboratory conditions) but also in running water from various sources (e.g., factory effluents, surface water, river waters). In the present investigation, the amphibians *Xenopus laevis* (anuran) and *Pleurodeles waltl* (urodele) were used because of their ecotoxicological relevance which has already been well established in a large array of studies on biomarkers such as induction of biotransformation enzyme activities (Bekaert et al., 2002; Gauthier et al., 2004), micronucleus (Ferrier et al., 1998) or DNA adduct formation (Bekaert et al., 2002), comet induction following DNA damage (Mouchet et al., 2005a,b, 2006b), or teratogenic malformations (Chenon et al., 2002; Prati et al., 2002; Bonfanti et al., 2004). Moreover, even though the toxic effects of Cd on aquatic organisms have been well documented, notably in amphibians (Pérez-Coll et al., 1999; Flament et al., 2003; Mounaji et al., 2003; James et al., 2004), less or no data on genotoxic effects are, to our knowledge, available for amphibians.

Among the numerous biomarkers of genotoxicity (DNA adducts, DNA strand breaks, chromosomal aberrations, sister chromatid-exchange, micronuclei, etc.) used to evaluate the genetic damage resulting from exposure to environmental pollutants, the present test for Cd was carried out with two different endpoints: micronucleus (micronucleus test, MNT) and primary DNA damage induction (Comet assay, CA) in two amphibian models (*Xenopus laevis* and *Pleurodeles waltl* larvae). The sensitivity of the two tests was compared.

The MNT detects the induction of micronuclei, which are the consequence of chromosome fragmentation and/or malfunction of the mitotic apparatus: thus, clastogenic compounds and spindle poisons both lead to an increase in the number of micronucleated cells. In aquatic vertebrates, the MNT has been carried out in different fish species (Das and Nanda, 1986; Metcalfe, 1988; Carrasco et al., 1990). Amphibians have also proved to be valuable biological models for the study of micronucleus induction in erythrocytes (Jaylet et al., 1986; Krauter et al., 1987; Jaylet and Zoll, 1990; Zoll et al., 1990; Gauthier, 1996; Ferrier et al., 1998; AFNOR, 2000; Djomo et al., 2000; Bekaert et al., 2002). The sensitivity and reliability of the MNT to detect chromosomic/genomic mutations makes it a good method to analyze the potential cytogenetic damage caused by pure substances (Grinfeld et al., 1986; Jaylet et al., 1986; Fernandez and Jaylet, 1987; Fernandez et al., 1989, 1993; Gauthier, 1996; Mouchet et al., 2005a).

The Comet assay (CA), as first described by Östling and Johanson (1984), and further developed by Singh et al. (1988) and Olive et al., (1990) is a sensitive method widely used for detecting DNA damage in individual cells (double- and single-strand breaks, alkali-labile sites, excision repair sites and crosslinks), induced by a variety of genotoxic agents such as industrial chemicals, biocides, agrochemicals, and pharmaceuticals (for general review articles see McKelvey et al., 1993; Fairbairn et al., 1995; Anderson and Plewa, 1998; Rojas et al., 1999; Speit and Hartmann, 1999; Tice et al., 2000; Hartmann et al., 2003). Numerous studies have demonstrated its capacity to detect low levels of DNA damage, its requirement for few cells, its low cost, and its speed of execution and analysis for assessing the genotoxicity of chemicals toward fish (Belpaeme et al., 1998; Devaux et al., 1998; Mitchelmore and Chipman, 1998; Flammarion et al., 2002; Winter et al., 2004), mussels (Nacci et al., 1996; Pavlica et al., 2001) and amphibians (Ralph et al., 1996; Clements et al., 1997; Ralph and Petras, 1998a,b; Mouchet et al., 2005a,b, 2006a,b,c).

MATERIALS AND METHODS

Amphibian (*Xenopus* and *Pleurodeles*) Maintenance and Breeding

Sexually mature *Xenopus* and *Pleurodeles* were provided by the Developmental Biology Department of Paul Sabatier University (Toulouse, France). The *Xenopus* males were injected with 400 IU of human chorionic gonadotropin (HCG) and the females with 700 IU of HCG to induce spawning. No hormonal induction was needed for *Pleurodeles* because of the natural laying period. The amphibian males and females were then placed together in normal tap water filtered through active charcoal at 22 ± 2 °C. Twelve hours later for *Xenopus*, the pair was separated and viable eggs were maintained in an aquarium also containing normal tap water filtered through active charcoal at 20–22 °C, until they reached a development stage appropriate for

experimentation (usually 3 weeks). For *Pleurodeles*, a few days after, viable eggs were maintained in a bowl containing 5–6 L of normal tap water filtered through active charcoal at 18 °C until the swimming stage. They were then transferred to an aquarium containing the same type of water at 20 ± 1 °C until they reached a development stage appropriate for experimentation (about 6 weeks after laying). The *Xenopus* larvae were fed every day on dehydrated aquarium fish food. The young *Pleurodeles* larvae were also fed every day on freshly hatched artemia (*Artemia* spec.), then, the food was switched to thawed *Chironomus* larvae. Each day, one third of the water was renewed before feeding.

Exposure Conditions

The experimental exposure conditions, basically the same for the MNT and CA, 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 either reconstituted water (RW, normal tap water filtered through active charcoal, to which were added nutritive salts [294 mg/L $CaCl_2 \cdot 2H_2O$, 123.25 mg/L $MgSO_4 \cdot 7H_2O$, 64.75 mg/L $NaHCO_3$, 5.75 mg/L KCl]), or mineral water (MW: 9.9 mg/L Ca^{2+}, 6.1 mg/L Mg^{2+}, 9.4 mg/L Na^+, 5.7 mg/L K^+, 8.4 mg/L Cl^-, 6.3 mg/L NO_3^-, 6.9 mg/L SO_4^{2-}, 65.3 mg/L HCO_3^{2-}, 30.0 mg/L Si, pH 7, Volvic®, Danone, France) at 22 ± 0.5 °C for *Xenopus* and at 20 ± 1 °C for *Pleurodeles*. Exposure began on larvae at stage 50 of the *Xenopus* development table (Nieuwkoop and Faber, 1956) characterized by the hind limb bud longer than broad, constricted at the base. For *Pleurodeles*, exposure began at stage 53 of the development table (Galien and Durocher, 1957) when the hind limbs present four well-formed digits with an outline of the fifth. The larvae were taken from the same hatch to reduce inter-animal variability. They were exposed in groups of 20 (100 mL/larvae) in 5-L glass flasks containing either a control medium—negative and positive control—or a test medium (different concentrations of Cd). The negative control was either RW or MW. Positive controls were systematically performed in each experiment to check both the responsiveness of the amphibian larvae and the correct operation of the experiment. The positive control for the MNT was benzo[a]pyrene (B[a]P, [50-32-8]; purity, ≥96.0%; Sigma France) at 0.125 mg/L (AFNOR, 2000). For the CA, the positive control was methyl methanesulfonate (MMS, [66-27-3]; purity, ~99%; Sigma France) at 1.56 mg/L (Mouchet et al., 2005a). B[a]P was dissolved in dimethylsulphoxide (DMSO [67-68-5], Sigma France) at a final concentration of 0.05%—previously shown to be nongenotoxic and nontoxic on *Xenopus*, (data not published)—before being added to the water. The concentrations of Cd as $CdCl_2$ were 0.25, 0.5, 1, 2, 10, 50 mg/L. Amphibian larvae were submitted to a 12 h light and 12 h dark cycle. *Xenopus* were fed every day on dehydrated aquarium fish food and *Pleurodeles* on thawed *Chironomus* larvae. The flasks were partially immersed in water baths to maintain their temperature at 22 ± 0.5 °C for *Xenopus* and 20 ± 1 °C for *Pleurodeles*. The media in control and exposed flasks were renewed daily.

Acute Toxicity

Acute toxicity to the larvae exposed to Cd in RW and MW was examined for 12 days by visual inspection: death and no growth (severe toxicity) and abnormal behavior, reduced size, diminished food intake (weak toxicity). Recording the toxicity data daily in treated larvae in MW ensured that genotoxicity tests were performed under conditions that were not acutely toxic.

Genotoxicity Assays

Genotoxicity was only assayed in MW. At the end of exposure, i.e., 12 days for the MNT and 1, 2, 4, 8, and 12 days for the CA, a blood sample was obtained from each anaesthetized larva by cardiac puncture with heparinized micropipettes (20% solution at 5000 IU/mL). For a given test (MNT or CA), larvae coming from the same group of exposure were used. Genotoxicity was assessed up to the concentrations at which signs of acute toxicity began to appear in the exposed larvae.

Micronucleus Test

The MNT was carried out according to the Standard AFNOR NF T90-325 procedure (AFNOR, 2000). At the end of the 12 days of exposure, a single smear of blood was prepared from each animal ($n = 15$). After fixing in methanol and staining with hematoxylin, the smears were screened under the microscope (oil immersion lens, 1500×). The number of erythrocytes that contained one micronucleus or more was determined in a total sample of 1000 erythrocytes per larva. Slides were blind scored by only one individual.

For each group of animals, the results (number of micronucleated erythrocytes per thousand, MNE ‰) obtained for the individual larvae were arranged in increasing order of magnitude. The medians and quartiles were then calculated. The statistical method used to compare the medians was based on the recommendations of Mc Gill et al. (1978) and consisted of determining the theoretical medians of samples of size n (where $n \geq 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times \mathrm{IQR}/\sqrt{n}$, where M is the median and IQR is the inter-quartile range (upper quartile–lower quartile). Under these conditions, the difference between the theoretical medians of the test groups and the

TABLE I. Results of the acute toxicity to *Xenopus* and *Pleurodeles* larvae exposed to cadmium (mg/L) in mineral water and reconstituted water for 12 days

Concentration (mg/L)	0	0.25	0.5	1	2	5	10	50
Xenopus								
MW	−	−	−	−	+ (until day 4)	++ (40% in 6 days)	++ (100% in 3 days)	++ (100% in 1 day)
RW	−	−	−	−	−	+ (from day 8)	++ (60% in 6 days)	++ (100% in 3 days)
Pleurodeles								
MW	−	−	−	−	−	+ (from day 2)	++ (100% in day 1)	++ (100% in day 1)
RW	−	−	−	−	−	−	++ (50% in 6 days)	++ (100% in 4 days)

%, percentage of deaths ($n = 20$ larvae); −, no toxicity to larvae; +, weak toxicity; ++, severe toxicity.

theoretical median of the negative control group is significant to within 95% certainty if there is no overlap.

Comet Assay

The CA was performed under alkaline conditions, according to the procedure described by Singh et al. (1988) and subsequently adapted to *Xenopus laevis* (Mouchet et al., 2005a) and *Pleurodeles waltl* larvae (Mouchet et al., 2006b). After cardiac puncture, erythrocyte viability was routinely determined using the Trypan blue exclusion test, and samples showing <90% viability were discarded (Collins, 2002). Sixty-five microliters of diluted blood in low-melting-point agarose (LMA, 1% in phosphate buffered saline PBS) were spread on precoated slides (normal melting agarose NMA, 0.8% in PBS) and covered with a coverslip (22*50). After cooling on ice and removing the coverslip, a third layer (90 μl of LMA 1% in PBS) was then added and allowed to solidify on ice. The slides were immersed in freshly prepared ice-cold lysing solution (4 °C, 2.5M NaCl, 0.1 M Na_2 EDTA, 0.01 M Tris, 1% Triton X-100, 10% DMSO, adjusted to pH 10 with NaOH) and left for 1 h. Lysis and subsequent steps were performed in the dark under dim red light. The slides were then transferred to a horizontal electrophoresis tank containing freshly prepared alkaline buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH >13) for 20 min at 4°C in order to allow the DNA to unwind. Electrophoresis was carried out in the same buffer at 4 °C for 20 min in *Xenopus* and 30 min in *Pleurodeles* (electric field of 20 V and 300 mA). The slides were washed twice in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min, and finally dehydrated in absolute ethanol for 5 min. Slide analysis was performed using a confocal microscope (LSM 410 inverted laser scanning microscope, Zeiss) at 40× magnification, after staining the slides with 0.05 mM ethidium bromide solution.

Quantitative assessment of DNA damage in erythrocyte nuclei was performed using the Komet 4.1 image analysis software (Kinetic Imaging Ltd), by measuring four parameters: extent tail moment (ETM, defined as the tail length weighted by the percentage of tail DNA), olive tail moment (OTM, product of the amount of DNA in the tail and the mean distance of migration in the tail, Olive et al., 1990), Tail DNA (percentage of DNA in the tail), and tail length (TL, length of the tail, distance between the head and the last DNA fragment). Slides were blind scored by only one individual. Thirty randomly selected cells were analysed on each slide. Comet images were randomly captured from the centre of the slide, at a constant depth in the gel, avoiding the edges of the slide and overlapping figures. Comets with completely fragmented DNA (hedgehog-like figures with no apparent head) that could not be measured by the image analysis system were not of course taken into account. Data from two slides per animal and two animals for each concentration (120 measurements per concentration tested) were analysed using the nonparametric Kolmogorov–Smirnov test (Mouchet et al., 2005a,b, 2006a,b). The following evolution coefficient was proposed in order to establish the time-course of DNA damage between two successive exposure times at a given concentration i of Cd (Mouchet et al., 2006b):

$$\begin{aligned} EC_{CA} = [[\{m(\text{concentration } i,\ \text{time } 2) - m(\text{concentration } i,\ \text{time } 1)\} - \{m(\text{neg.control, time } 2) \\ - m(\text{neg.control, time } 1)\}]/\{m(\text{concentration } i,\ \text{time } 1) - m(\text{neg.control, time } 1)\}] \times 100 \end{aligned} \quad (1)$$

where m(concentration i, time 2), m(concentration i, time1), and m(neg.control, time 2), m(neg.control, time 1) represent the mean values of the parameter computed for a given concentration i of Cd, and the corresponding negative control, at the two exposure times considered.

RESULTS

Acute Toxicity

Xenopus Laevis

Cd was found to be severely toxic to *Xenopus* larvae exposed from 2 to 50 mg/L of Cd in MW (Table I). 100%

TABLE II. Results of the *Xenopus* and *Pleurodeles* micronucleus assay

Concentration (mg/L)	NC	PC	0.25	0.5	1	2
Xenopus						
Lower quartile	1	9	1	2	3	5.5
Median	2	12	1	2	4	7
Confidence limit	(1.19–2.81)	(10.32–13.68)	(0.59–1.41)	(2–2)	(2.58–5.42)	(5.99–8.01)
Upper quartile	3	13	2	2	6.5	8
Results		+	−	−	−	+
Number of animals	15	15	15	15	15	15
Pleurodeles						
Lower quartile	21.5	78.5	9	7	13	12
Median	23	85	9	9.5	15	18
Confidence limit	(21.99–24.01)	(80.54–89.46)	(6.91–11.09)	(7.51–11.87)	(12.16–17.84)	(15.58–20.42)
Upper quartile	24	89.5	13	11	20	24
Results		+	−	−	−	−
Number of animals	15	15	15	15	15	15

Frequencies of micronucleated erythrocytes per 1000 cells in larvae exposed to cadmium (mg/L) for 12 days in mineral water. Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand) and their 95 % confidence limits. NC, negative control; PC, positive control; −, nongenotoxic concentration compared to the negative control group (Mac Gill et al., 1978); +, genotoxic concentration compared to the negative control group (Mac Gill et al., 1978).

lethality was obtained for 10 and 50 mg/L on days 1 and 3, respectively. 40% lethality was also recorded at 5 mg/L of Cd in MW on day 6. Cd appeared weakly toxic to *Xenopus* larvae exposed to 2 mg/L until day 4. No signs of acute toxicity were observed for *Xenopus* tadpoles exposed to lower Cd-concentrations in MW (0.25, 0.5, and 1 mg/L).

TABLE III. Results of the *Xenopus* comet assay: mean Tail DNA, ETM, OTM and TL values in larvae exposed from 1 to 12 days to cadmium in mineral water

			Concentrations of Cadmium (mg/L)			
Exposure time (days)	Parameter	NC	0.25	0.5	1	PC_{MMS}
1	Tail DNA	3.11 ± 0.81	3.51 ± 0.79*	6.92 ± 1.97*	4.44 ± 0.75*	17.54 ± 2.66*
	ETM	0.72 ± 0.27	1.44 ± 0.43*	3.37 ± 1.03*	1.69 ± 0.47*	10.75 ± 2.38*
	OTM	0.74 ± 0.22	0.93 ± 0.22*	1.98 ± 0.44*	1.15 ± 0.22*	6.14 ± 0.92*
	TL	17.9 ± 2.3	29.62 ± 2.83*	33.76 ± 3.85*	29.38 ± 2.53*	56.65 ± 5.58*
2	Tail DNA	2.73 ± 0.57	2.88 ± 0.49	10.91 ± 1.79*	5.06 ± 1.24*	18.31 ± 2.78*
	ETM	0.69 ± 0.16	0.87 ± 0.2	6.33 ± 1.37*	2.03 ± 0.76*	10.28 ± 2.16*
	OTM	0.67 ± 0.15	0.7 ± 0.12	3.64 ± 0.61*	1.35 ± 0.4*	6.49 ± 1*
	TL	22.55 ± 2.08	24.35 ± 2.34*	44.68 ± 3.98*	25.99 ± 2.75*	51.18 ± 5.51*
4	Tail DNA	1.89 ± 0.43	3.58 ± 1.44*	2.31 ± 0.44	2.25 ± 0.43	18.1 ± 3.57*
	ETM	0.48 ± 0.15	1.33 ± 0.63*	0.76 ± 0.21	0.83 ± 0.25	11.01 ± 2.63*
	OTM	0.43 ± 0.1	0.87 ± 0.28*	0.59 ± 0.12	0.59 ± 0.13	5.4 ± 0.82*
	TL	19.5 ± 2.63	25.76 ± 2.74*	22.95 ± 2.81*	22.66 ± 3.09*	55.33 ± 4.47*
8	Tail DNA	1.78 ± 0.47	1.61 ± 0.25**	2.1 ± 0.4*	3.46 ± 0.65*	11.76 ± 1.44*
	ETM	0.42 ± 0.15	0.41 ± 0.1	0.55 ± 0.16	1.02 ± 0.25*	6.03 ± 1.08*
	OTM	0.38 ± 0.09	0.36 ± 0.06	0.51 ± 0.1	0.87 ± 0.18*	4.01 ± 0.49*
	TL	18 ± 2.91	22.47 ± 2.59*	19.23 ± 2.30	22.23 ± 2.17*	48.43 ± 4.27*
12	Tail DNA	3.13 ± 2.23	2.9 ± 0.54	1.68 ± 0.41**	1.7 ± 0.32**	12.46 ± 1.62*
	ETM	0.87 ± 0.79	1.04 ± 0.28*	0.38 ± 0.16**	0.36 ± 0.1**	5.5 ± 0.94*
	OTM	0.53 ± 0.29	0.75 ± 0.14*	0.39 ± 0.13**	0.37 ± 0.07**	4.19 ± 0.57*
	TL	21.53 ± 3.51	27.31 ± 2.52*	17.91 ± 1.84**	16.07 ± 2.01**	41.05 ± 3.34*

This table presents the calculated mean values of Tail DNA (%DNA in the tail), ETM (extent tail moment), OTM (olive tail moment) and TL (tail length) followed by their 95% confidence limits. The notation "*" indicates an increase of DNA damage, relative to the negative control, confirmed by a Kolmogorov-Smirnov test ($p = 0.05$). "**" indicates a decrease of DNA damage, relative to the negative control, confirmed by a Kolmogorov-Smirnov test ($p = 0.05$). NC, negative control group; PC_{MMS}, positive control group (MMS to 1.56 mg/L).

In RW, Cd was acutely toxic to *Xenopus* larvae from 5 to 50 mg/L of Cd (Table I). 100% and 60% lethality were obtained for 50 and 10 mg/L, respectively, in 3 and 6 days, respectively. Cd appeared weakly toxic to *Xenopus* larvae exposed to 5 mg/L from the day 8. Cd was not toxic to *Xenopus* tadpoles exposed to lower Cd-concentrations in RW (0.25, 0.5, 1, and 2 mg/L).

Pleurodeles Waltl

In the same way, Cd was acutely toxic to *Pleurodeles* at 10 and 50 mg/L of Cd in MW (Table I). 100% lethality was observed at both these concentrations on day 1. Cd appeared weakly toxic to *Pleurodeles* larvae exposed to 5 mg/L from the day 2. No signs of acute toxicity were observed for *Pleurodeles* tadpoles exposed to lower Cd-concentrations in MW (0.25, 0.5, 1, and 2 mg/L).

In RW, Cd was severely toxic to *Pleurodeles* at 10 and 50 mg/L (Table I). 50% and 100% lethality were observed at both these concentrations in 6 and 4 days, respectively. Cd was not toxic to *Pleurodeles* tadpoles exposed to lower Cd-concentrations in RW (0.25, 0.5, 1, 2, and 5 mg/L).

Micronucleus Test

The median value of micronucleated erythrocytes per thousand (MNE ‰) was 2 ± 0.81 (Table II) and 23 ± 1.01 (Table II) for the negative control in the *Xenopus* and *Pleurodeles* MNT, respectively. The positive control showed a significantly higher MNE ‰ compared to the negative one in both MNT: 12 ± 1.68 (Table II) and 85 ± 4.46 (Table II) for *Xenopus* and *Pleurodeles* MNT, respectively.

Xenopus Laevis

Results of the *Xenopus* MNT showed that 2 mg/L of Cd induced a significant genotoxic response (Table II). Indeed, the result is significant at a median value of 8 for a 95% confidence limit. The lowest Cd-concentrations (0.25, 0.5, and 1 mg/L) were nongenotoxic to *Xenopus* larvae. Nevertheless, a dose-response relationship was apparent between the four Cd-concentrations.

Pleurodeles Waltl

Results of the *Pleurodeles* MNT showed no genotoxicity irrespective of the Cd-concentration: 0.25, 0.5, 1, and 2 mg/L (Table II). Moreover, no clear dose-response relationship was apparent.

Comet Assay

Results of PC_{MMS} showed a significant genotoxic effect of MMS as positive control, compared with negative control, for both CA using *Xenopus* and *Pleurodeles*, at all exposure times (1, 2, 4, 8, and 12 days).

TABLE IV. Results of the *Xenopus* comet assay: dose effect relationships in larvae exposed for 1 to 12 days to cadmium in mineral water

Exposure time (days)	Parameter	Cadmium concentration (mg/L)	
		0.25 vs 0.5	0.5 vs 1
1	Tail DNA	*	**
	ETM	*	**
	OTM	*	**
	TL	*	**
2	Tail DNA	*	**
	ETM	*	**
	OTM	*	**
	TL	*	**
4	Tail DNA	–	–
	ETM	–	–
	OTM	–	–
	TL	**	–
8	Tail DNA	*	*
	ETM	*	*
	OTM	*	*
	TL	**	*
12	Tail DNA	**	–
	ETM	**	–
	OTM	**	–
	TL	**	**

The notation "*" indicates a positive significant dose-effect relationship, confirmed by a Kolmogorov-Smirnov test ($p = 0.05$). "**" indicates a negative significant dose-effect relationship, confirmed by a Kolmogorov-Smirnov test ($p = 0.05$). "–" indicates a nonsignificant dose-effect relationship. "vs," a given concentration is compared versus another one, based on the calculated mean values of Tail DNA (%DNA in the tail), ETM (extent tail moment), OTM (olive tail moment), and TL (tail length).

Xenopus Laevis

The results of the *Xenopus* CA (Table III) showed a genotoxic effect of Cd after one day of exposure, for all Cd concentrations (0.25, 0.5, and 1 mg/L), whatever the parameter. After 2 days of exposure, the results showed a genotoxic effect of Cd for all concentrations, irrespective of the parameter except for Tail DNA, ETM, and OTM at 0.25 mg/L. In the same way, Cd was genotoxic after 4 days of exposure, whatever the concentration and the parameters, except for 0.5 and 1 mg/L using Tail DNA, ETM, and OTM. After 8 days of exposure, Cd induced genotoxic effects at 1 mg/L, whatever the parameter, and at 0.5 and 0.25 mg/L using Tail DNA and TL, respectively. In contrast, 0.2 mg/L of Cd induced a significant decrease in DNA damage using Tail DNA. In the same way, after 12 days of exposure, 0.5 and 1 mg/L of Cd induced a significant decrease in DNA damage, irrespective of the parameter, whereas 0.25 mg/L

TABLE V. Results of the *Xenopus* comet assay: evolution coefficients (EC_{CA}) of tail DNA, ETM, OTM, and TL in larvae exposed for 1 to 12 days to cadmium in mineral water

			Cadmium concentration (mg/L)		
Exposure time (days)	Parameter	NC	0.25	0.5	1
1–2	Tail DNA	−12.15	−64.38	+114.96*	+75.34*
	ETM	−5.24	−74.82*	+112.97*	+38.98*
	OTM	−10.25	−83.19	+140.85*	+68.90*
	TL	+25.97*	−84.63*	+39.52*	−70.00*
2–4	Tail DNA	−30.80*	+1093.33*	−94.86*	−84.80*
	ETM	−29.67*	+365.19	−95.11*	−74.33*
	OTM	−36.01*	+1282.29	−94.64*	−76.38*
	TL	−13.53*	+247.69	−84.41*	−8.23*
4–8	Tail DNA	−5.90*	−109.99*	−23.95	+374.71*
	ETM	−13.38	−101.02*	−51.10	+75.76*
	OTM	−10.57	−104.01*	−16.37	+200.10*
	TL	−7.69	−28.63*	−64.25*	+33.77*
8–12	Tail DNA	+76.63*	+36.44*	−553.61*	−185.26*
	ETM	+107.61*	−2136.89*	−458.70*	−184.29*
	OTM	+38.73*	−1333.80*	−206.63*	−132.77*
	TL	+19.63	+29.29*	−393.92*	−229.39*
1–12	Tail DNA	+0.48	−157.95	−138.13*	−207.87*
	ETM	+19.82*	−75.71	−118.28*	−152.85*
	OTM	−28.75*	+15.06	−111.52*	−139.24*
	TL	+20.30*	−50.71	−122.86*	−147.64*

These evolution coefficients are calculated as described in Eq. 1. Therefore, a positive value corresponds to increased DNA damage (and vice versa) between two successive exposure times. "*" indicates that the evolution of DNA damage between two successive exposure times is significant (Kolmogorov–Smirnov test, $p = 0.05$). Tail DNA, %DNA in the tail; ETM, extent tail moment; OTM, olive tail moment; TL, tail length.

induced a significant increase in DNA damage, whatever the parameter, except for tail DNA.

A positive significant dose-effect relationship was observed with 0.25 and 0.5 mg/L of Cd (Table IV) after 1, 2, and 8 days of exposure, (except for TL after 8 days), whereas a negative significant dose-effect relationship was observed with the same concentrations after 4 days and 8 days using TL parameter and after 12 days, whatever the parameter. With 0.5 and 1 mg/L, a negative significant dose-effect relationship was noted after 1 and 2 days of exposure, whatever the parameter and after 12 days of exposure using TL parameter. A positive significant dose-effect relationship was noted after 8 days of exposure, irrespective of the parameter. For the other times and parameters, no significant dose-effect relationship was recorded.

The evolution coefficients (Table V) showed that levels of DNA damage increased or decreased according to the exposure time for a given concentration, including for the negative control. The overall change for the negative control between days 1 and 12 indicated a significant increase in the values of the ETM and TL parameters after Cd exposure, unlike OTM (significant decrease). For Cd-exposure, the overall change in DNA damage between days 1 and 12 indicated a significant decrease in the values of all the parameters at 0.5 and 1 mg/L of Cd, unlike at 0.25 mg/L.

Pleurodeles Waltl

Results of the *Pleurodeles* CA (Table VI) showed a genotoxic effect of Cd after 1 and 12 days, at all concentrations (0.5, 1, and 2 mg/L), whatever the parameter. After 2 days of exposure, Cd induced significant DNA damage at 2 mg/L only, whatever the parameter, whereas no significant DNA damage was observed at 0.5 and 1 mg/L of Cd, whatever the parameter, except for 1 mg/L using TL (significant decrease of DNA damages). After 4 days of exposure, Cd induced significant DNA damage, at all concentrations and all parameters, except for 1 mg/L and 2 mg/L with TL (significant decrease in DNA damage). After 8 days, Cd induced genotoxic effects only at 1 mg/L using the OTM parameter.

A positive significant dose-effect relationship (Table VII) was obtained for 0.5 versus 1 mg/L after 8 days of exposure only, whatever the parameter and after 12 days of exposure using only the TL parameter. With these concentrations, a significant negative dose-effect relationship was obtained after 2 days using parameter TL and after 4 days using parameters ETM and TL. When considering 1 mg/L versus 2 mg/L, a positive significant dose-effect relationship was obtained only after 1 day using OTM and after 2 days whatever the parameter. In contrast, a significant negative dose-effect relationship was obtained after 4 and 8 days,

TABLE VI. Results of the *Pleurodeles* comet assay: mean tail DNA, ETM, OTM and TL values in larvae exposed for 1 to 12 days to cadmium in mineral water

			Concentrations of cadmium (mg/L)			
Exposure time (days)	Parameter	NC	0.5	1	2	PC_{MMS}
1	Tail DNA	0.49 ± 0.12	0.98 ± 0.25*	0.89 ± 0.23*	0.91 ± 0.17*	5.03 ± 0.88*
	ETM	0.06 ± 0.03	0.2 ± 0.08*	0.13 ± 0.05*	0.14 ± 0.04*	2.23 ± 0.51*
	OTM	0.15 ± 0.04	0.27 ± 0.07*	0.23 ± 0.06*	0.27 ± 0.05*	2.18 ± 0.37*
	TL	10.25 ± 2.95	12.26 ± 1.86*	10.85 ± 1.58*	12.03 ± 1.61*	40.45 ± 3.82*
2	Tail DNA	0.74 ± 0.16	0.95 ± 0.25	0.8 ± 0.17	1.03 ± 0.22*	5.21 ± 0.84*
	ETM	0.13 ± 0.04	0.16 ± 0.05	0.11 ± 0.04	0.21 ± 0.06*	2.73 ± 0.59*
	OTM	0.22 ± 0.05	0.28 ± 0.08	0.22 ± 0.05	0.31 ± 0.06*	2.26 ± 0.36*
	TL	13.77 ± 2.45	13.2 ± 1.79	11.28 ± 1.95**	16.17 ± 2.6*	47.27 ± 3.35*
4	Tail DNA	0.68 ± 0.17	1.11 ± 0.22*	1.22 ± 0.28*	1.05 ± 0.26*	7.66 ± 1.55*
	ETM	0.13 ± 0.05	0.28 ± 0.08*	0.23 ± 0.07*	0.15 ± 0.05*	4.29 ± 1.15*
	OTM	0.19 ± 0.04	0.36 ± 0.07*	0.35 ± 0.08*	0.27 ± 0.07*	3.68 ± 0.71*
	TL	16.48 ± 2.8	19.77 ± 2.71*	14.38 ± 1.65	11.63 ± 1.28**	48.4 ± 4.72*
8	Tail DNA	0.82 ± 0.17	0.73 ± 0.12	0.95 ± 0.14	0.78 ± 0.13	10.38 ± 2.37*
	ETM	0.13 ± 0.05	0.1 ± 0.02	0.16 ± 0.03	0.13 ± 0.04	8.41 ± 2.67*
	OTM	0.22 ± 0.04	0.2 ± 0.03	0.27 ± 0.04*	0.22 ± 0.03	5.33 ± 1.32*
	TL	12.92 ± 1.77	12.43 ± 1.31	14.6 ± 1.52	12.51 ± 1.45	63.95 ± 5.95*
12	Tail DNA	0.5 ± 0.14	0.9 ± 0.18*	0.87 ± 0.22*	0.75 ± 0.13*	10.26 ± 1.54*
	ETM	0.06 ± 0.02	0.14 ± 0.04*	0.16 ± 0.06*	0.1 ± 0.03*	7.57 ± 1.58*
	OTM	0.14 ± 0.04	0.25 ± 0.05*	0.26 ± 0.07*	0.2 ± 0.03*	5.35 ± 0.87*
	TL	9.78 ± 1.84	11.41 ± 1.37*	12.91 ± 1.58*	10.9 ± 1.14*	67.03 ± 4.52*

This table presents the calculated mean values of tail DNA (%DNA in the tail), ETM (extent tail moment), OTM (olive tail moment), and TL (tail length) followed by their 95% confidence limits. The notation "*" indicates an increase of DNA damage, relative to the negative control, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$). The notation "**" indicates a decrease of DNA damage, relative to the negative control, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$). NC, negative control group; PC_{MMS}, positive control group (MMS to 1.56 mg/L).

whatever the parameter and after 12 days using TL parameter only.

Evolution coefficients (Table VIII) showed that levels of DNA damage increased or decreased according to the exposure time for a given concentration, including for the negative control. The overall change for the negative control between days 1 and 12 indicated that the level of DNA damage remained unchanged, whatever the parameter. The overall change in DNA damage between days 1 and 12 of Cd-exposure indicated a significant decrease in the values of all the parameters except for ETM, OTM, and TL at 1 mg/L (significant increase).

DISCUSSION

Acute Toxicity

Cd was acutely toxic to the amphibian larvae depending on the concentrations and the nature of the exposure water. Toxic concentrations of Cd in MW ranged from 2 mg/L in *Xenopus* and 5 mg/L in *Pleurodeles*, to 50 mg/L for the two species. They seemed to be higher for amphibian larvae exposed in RW than in MW; indeed, the toxic concentrations ranged from 5 mg/L to 50 mg/L in *Xenopus*, and from 10 mg/L to 50 mg/L in *Pleurodeles*. In the present investigation, the Ca^{2+} concentration was about 700 times higher in RW than in MW. Consequently, the greater ion concentrations in RW may limit the sensitivity of larvae by inhibiting the toxic potential of Cd. In fact, the toxicity of certain metals, in relation with their bioavailability, is strongly correlated to their chemical speciation (Chapman and McCrady, 1977; Spokes et al., 1996). In particular, interactions between bivalent metal and aqueous calcium ion is especially now well known such as mimetic action and competition between metal ions and calcium (Wood, 1983; Pärt et al., 1985; Wicklund and Runn, 1988; Freda, 1991; Playle et al., 1993a,b; Playle, 1998; Richards et al., 1999; Meinelt et al., 2001; Perceval et al., 2002). *Xenopus* larvae seemed to be more sensitive than *Pleurodeles* to the acute toxicity of Cd in both kinds of water (MW and RW). Their different sensitivities may be partly linked to the mechanisms of Cd uptake at the gills via the Ca^{2+}-transport pathway (Wood, 2001) competitively inhibiting the high-affinity Ca^{2+}-ATPase in *Pleurodeles*, which have large external gills in contact with the exposure medium.

Micronucleus Induction

Cd appeared to be nongenotoxic in *Pleurodeles* and genotoxic at the highest concentration in *Xenopus*. Although cadmium is suspected to have an effect on the mitotic

TABLE VII. Results of the *Pleurodeles* comet assay: dose-effect relationships in larvae exposed for 1 to 12 days to cadmium in mineral water

Exposure time (days)	Parameter	Cadmium concentration (mg/L)	
		0.5 vs 1	1 vs 2
1	Tail DNA	–	–
	ETM	–	–
	OTM	–	*
	TL	–	–
2	Tail DNA	–	*
	ETM	–	*
	OTM	–	*
	TL	**	*
4	Tail DNA	–	–
	ETM	**	**
	OTM	–	**
	TL	**	**
8	Tail DNA	*	**
	ETM	*	**
	OTM	*	**
	TL	*	**
12	Tail DNA	–	–
	ETM	–	–
	OTM	–	–
	TL	*	**

The notation "*" indicates a positive significant dose-effect relationship, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$). "**" indicates a negative significant dose-effect relationship, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$). "–" indicates a nonsignificant dose-effect relationship. "vs," a given concentration is compared versus another one, based on the calculated mean values of tail DNA (%DNA in the tail), ETM (extent tail moment), OTM (olive tail moment), and TL (tail length).

spindl and is known for its likely carcinogenic, mutagenic and teratogenic activity (IARC, 1987; 1993a,b), cytogenetic bioassay data are conflicting (Forni, 1992; Hartwig, 1994; Verougstraete et al., 2002).

Despite the lack of cytogenetic damage related to the exposure of *Pleurodeles* larvae, a significant increase in the frequency of micronuclei in *Xenopus* larvae was observed at 2 mg/L. Therefore, it appears that the amphibian models are also probably different in terms of micronucleus induction. Different sensitivity of the amphibians can occur depending on how the animals are exposed to the contaminant. However, only limited comparisons between models is possible because of their different biological characteristics such as their chromosomes (small, $2n = 36$ in *Xenopus* and big, $2n = 24$ in *Pleurodeles*), genomic size (3.1 pg in *Xenopus* and 19.5 pg in *Pleurodeles* (Fernandez et al., 1993), and because of the food they eat, the temperature of exposure, etc.

Primary DNA Damage as Comet Induction

Primary DNA damage as comet induction was significantly observed in *Xenopus* and *Pleurodeles* larvae. While the mechanism underlying the genotoxic effects is not well known, several reports have suggested that Cd induces DNA strand breaks and chromosomal aberrations, even if its mutagenic potential is rather weak (Hartwig, 1994; Waisberg et al., 2003). Cadmium is also known to induce genotoxicity as an indirect effect, via oxidative stress (Devi et al., 2001; Filipic and Hei, 2004), DNA binding (Hassoun and Stohs, 1996), or inhibition of DNA repair activities (Hartwig, 1998; Lützen et al., 2004). In the present study, Cd led to DNA damage in *Xenopus* at each exposure time and at all concentrations except after 12 days at 0.5 and 1 mg/L of Cd for which significant reduction of these DNA damage was observed, compared to the negative control. In *Pleurodeles*, the genotoxic effects of Cd were less clear, depending on the exposure time and the Cd-concentrations, because of the probable constant balancing occurring between repair and damage. In the same way, significantly reduced DNA damage was obtained over the course of the exposure in both amphibian larvae. Such observations have already been reported in previous investigations (Mouchet et al., 2005a,b, 2006a,b). In the present study, the reduction in DNA damage observed in both amphibian larvae for a given exposure time, and over the course of the exposure, could have stemmed from different cellular processes, including DNA repair, detoxification, and adaptive responses to repeated doses. Indeed, it is well established that repair processes are genetically controlled (Moustacchi, 2000), each repair system preferentially acting on a particular kind of damage (Friedberg et al., 1995).

Analysis of the CA results reveals that negative controls often exhibited significant background levels of strand breaks, which were dependent on the time of exposure. This observation is in agreement with previous studies and already discussed (Mouchet et al., 2005a,b, 2006a,b). The introduction of the evolution coefficient enabled us to consider only the part of the DNA damage that was related to the treatment.

For a given biological model, with regard to the four parameters, genotoxic responses were sometimes different. This result, confirming also previous study (Mouchet et al., 2005a,b, 2006a,b), emphasizes the importance of the number of parameters used in evaluating pollutant genotoxicity. Indeed, at least two parameters must be used simultaneously.

Comparison Between CA and MNT

Because of the low acute toxicity in *Xenopus* larvae at 2 mg/L of Cd until day 4, the Cd concentrations analyzed were 0.25, 0.5, and 1 mg/L, and 2 mg/L was not studied in CA. The genetic results obtained in the present evaluation show a significant increase in the frequency of micronuclei in *Xenopus* larvae exposed to only 2 mg/L of Cd, whereas significant primary DNA damage was found whatever the

TABLE VIII. Results of the *Pleurodeles* comet assay: evolution coefficients (EC_{CA}) of tail DNA, ETM, OTM, and TL in larvae exposed for 1 to 12 days to cadmium in mineral water

			Cadmium concentration (mg/L)		
Exposure time (days)	Parameter	NC	0.5	1	2
1–2	Tail DNA	+50.76*	−57.40	−85.10	−31.18
	ETM	+78.86*	−68.71	−110.41	+34.05*
	OTM	+52.92*	−53.40	−101.95	−26.31
	TL	+34.30*	−128*	−513.88	+35.21*
2–4	Tail DNA	−7.25	+103.71*	+794.59*	−84.80
	ETM	+10.95	+265.27*	−1460.91*	−75.95
	OTM	−12.53	+172.13*	−8819.04*	−76.38
	TL	+19.73*	−680.88*	−15.43*	−302.43*
4–8	Tail DNA	+20*	−121.37*	−76.93	+23.94*
	ETM	+4.67	−121.12*	−75.92	−104.94
	OTM	+14.16*	−114.74*	−66.15	+200.10*
	TL	−21.63*	−114.68*	−64.25	−91.59
8–12	Tail DNA	−38.91*	−531.21	+200.13*	−676.55
	ETM	−54.34*	−331.6	+309.12*	−3750
	OTM	−35.03*	−556.35	+131.38*	−132.77
	TL	−24.25*	−436.20*	+85.64*	−373.46
1–12	Tail DNA	+2.62	−20.02*	−7.72*	−41.64*
	ETM	−5.15	−44.07*	+39.64*	−41.79*
	OTM	+0.17	−14.65*	+32.74*	−139.23*
	TL	−4.55	−19.08*	+420.83*	−37.08*

These evolution coefficients are calculated as described in Eq 1. Therefore, a positive value corresponds to increased DNA damage (and vice versa) between two successive exposure times. "*" indicates that the evolution of DNA damage between two successive exposure times is significant (Kolmogorov–Smirnov test, $p = 0.05$); tail DNA, %DNA in the tail; ETM, extent tail moment; OTM, olive tail moment; TL, tail length.

exposure time and the Cd-concentration (except for 0.5 and 1 mg/L after 12 days of exposure), depending on the parameter studied. Moreover, even though Cd did not lead to the induction of any micronuclei in *Pleurodeles* at any concentration, it did induce significant primary DNA damage depending on the concentration and the parameter studied. This finding may first suggest a lack of clastogenic and/or aneugenic effects in larvae related to the Cd concentrations studied. Indeed, genetic endpoints are different. The *in vivo* MNT in amphibians (AFNOR, 2000) detects chromosomal and/or genomic mutations (chromosomal damage and/or alteration of mitotic spindles), whereas alkaline CA detects primary DNA damage, expressed as single- and double-strand breaks, alkali-labile sites that are expressed as single-strand breaks and single-strand breaks associated with incomplete repaired excision (sites present at the time of cell lysis). Cd exposure led to significant DNA damage measured through CA and not through MNT in *Xenopus* and *Pleurodeles* after 12 days of exposure at the same concentrations. This result suggests that Cd-concentrations which induce primary DNA lesions cannot give rise to fixed mutations, assuming that single-strand breaks do not necessarily induce chromosomic damage resulting in micronucleus formation. These results may also emphasize the repair capacity of these organisms. Indeed, primary DNA lesions induced by Cd-concentrations which did not lead to the formation of micronuclei (0.25, 0.5, and 1 mg/L in *Xenopus* and 0.5, 1, and 2 mg/L in *Pleurodeles*) were repaired by the induction of genetic mechanisms, since they did not persist after one mitotic cycle. However, with 2 mg/L, Cd saturation of these repair systems may be reached in *Xenopus*. So, it is not surprising that concentrations of genotoxicant can induce single-strand breaks, as revealed by the CA, without necessarily inducing micronucleus formation because of repair mechanisms, as already observed in a previous study on captan exposure (Mouchet et al., 2006b). Moreover, *Xenopus* larvae are known to establish an effective detoxification mechanism, from the first day of exposure, theoretically capable of "minimizing" the toxic effects of cadmium via production of metallothioneins (MTs) (Mouchet et al., 2006c). MTs may, at least in part, contribute to limiting micronucleus formation due to direct Cd effects. In this previous study, Cd was shown to induce micronuclei at relatively low concentrations (2, 10, and 30 μg Cd/L) in *Xenopus* larvae in the same exposure conditions (AFNOR, 2000); MT induction was probably insufficient to inhibit the clastogenic effects of cadmium at relatively low concentrations and inhibit finally the micronucleus induction. In contrast, the present results suggest that the genotoxic mechanism of Cd action depends on the Cd concentration, the capacity of detoxification processes, and also on other pathways for exerting deleterious effects

on DNA. In the previous study, gene expression levels have also been investigated by quantitative real-time RT-PCR in liver and in the remaining tissues of larvae exposed to relatively low concentrations. For instance, genes participating in some other Cd-detoxification mechanisms to inhibit micronucleus induction, such as the *tap* gene (belonging to the ABC transporter family), have been found to be not expressed. Genes involved in DNA repair, such as the growth-arrest-DNA-damage (*gadd*), the *mutL* and the *rad51* genes, were found to be down-regulated at 30 μg Cd/L, and up-regulated at 10 μgCd/L, suggesting that the DNA damage generated by Cd can be repaired when exposure concentrations are low, whereas Cd seems to inhibit DNA repair mechanisms at higher concentrations. Genes encoding for antioxidant enzymes like *sodMt* and *coxI* were induced in larvae, indicating that Cd contamination generated oxidative stress in certain cells, depending on the tissues analysed.

In the present study, the CA applied to amphibian larvae for assessing genotoxicity of Cd seems to be a more sensitive method than the normalized MNT (AFNOR, 2000) since genotoxic Cd-effects were preferentially established though CA. Genotoxic responses to Cd can be measured from the first day of exposure with the CA, in comparison with the 12-day exposure required by the MNT in the standardized procedure (AFNOR, 2000). Taking these results into account and because of their different endpoints and response times, both tests carried out on amphibian larvae could be proposed in a complementary manner in a battery test system.

The studied concentrations in CA and MNT from 250 μg Cd/L to 2 mg Cd/L are higher than mean concentrations measured in natural contaminated waters. Environmental Cd-concentrations such as 2, 10, and 30 μg Cd/L (Mouchet et al., 2006c) are relevant for evaluating the Cd-stress in "chronic conditions". In the same way, higher Cd-concentrations can be considered relevant for evaluating this stress in "acute conditions" since important contaminated sediment and sludge stocks are accumulated in contaminated sites and would represent additional and potential Cd sources due to remobilization by floods, violent storms, dredging, or road and urban development.

CONCLUSION

Summing up, Cd could induce adverse effects in aquatic species. In amphibians, lethal concentrations of Cd may cause the immediate death of animals, while sublethal concentrations may increase the genotoxic potential of Cd and influence vulnerability (predation, fertilization, behavior, etc.). In amphibians, contaminants are of major concern because many populations are declining dramatically and the death of entire populations occurs increasingly frequently, in some cases due to anthropogenic changes in the environment. One of the key functions of such biomarkers (micronucleus and comets) is to provide an "early warning" signal of significant biological effects (changes at the genetic/molecular level) with suborganismic (molecular, biochemical, and physiological) responses preceding those occurring at higher levels of biological organization such as cellular, tissue, organ, whole-body levels, and in line at population level. Biomarkers are also well suited as rapid and effective screening assays to assess "hot spots" of contamination. In this way, the use of the MNT and CA may provide an important tool for the prediction of the potential long-term effects in amphibians in the environment.

Nevertheless, no increase in cytogenetic damage was found when evaluated by the MNT in *Pleurodeles* larvae, but an increase was found with 2 mg/L of Cd in *Xenopus* tadpoles. Moreover, genotoxic responses were observed in the form of primary DNA damage in both species. Considering that (i) the induction of DNA damage measured by the two bioassays stems from different mechanisms, and (ii) their different response-times, the CA could be carried out as a relevant complementary method to assess the genotoxicity of both pure substances and complex contaminated matrices, in biomonitoring studies using amphibian larvae.

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