Comparative study of the comet assay and the micronucleus test in amphibian larvae (Xenopus laevis) using benzo(a)pyrene, ethyl methanesulfonate, and methyl methanesulfonate: establishment of a positive control in the amphibian comet assay

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Comparative Study of the Comet Assay and the Micronucleus Test in Amphibian Larvae (*Xenopus laevis*) Using Benzo(*a*)pyrene, Ethyl Methanesulfonate, and Methyl Methanesulfonate: Establishment of a Positive Control in the Amphibian Comet Assay

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ABSTRACT: The present investigation explored the potential use of the comet assay (CA) as a genotoxicity test in the amphibian *Xenopus laevis* and compared it with the French standard micronucleus test (MNT). Benzo[*a*]pyrene (B[*a*]P), methyl methanesulfonate (MMS), and ethyl methanesulfonate (EMS) were used as model compounds for assessing DNA damage. Damage levels were measured as DNA strand breaks after alkaline electrophoresis of nuclei isolated from larval amphibian erythrocytes using the CA in order to establish a positive control for further ecotoxicological investigations. The results led to the selection of MMS as a positive control on the basis of the higher sensitivity of *Xenopus laevis* to this compound. The CA and MNT were compared for their ability to detect DNA damage with the doses of chemical agents and exposure times applied. EMS and MMS were shown to increase micronucleus and DNA strand break formation in larval erythrocytes concurrently. However, B[*a*]P increased micronucleus formation but not that of DNA strand breaks. Time–dose experiments over 12 days of exposure suggest that the CA provides an earlier significant response to genotoxicants than does the MNT. In *Xenopus* the CA appears to be a sensitive and suitable method for detecting genotoxicity like that caused by EMS and MMS. It can be considered a genotoxicity-screening tool. The results for B[*a*]P show that both tests should be used in a complementary manner on *Xenopus.* Environ Toxicol 20: 74–84, 2005.

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INTRODUCTION

The presence of pollutants exhibiting genotoxic activity in freshwater and their effects on ecosystems and human health is an area of increasing concern in industrialized countries. Whether the origin of the pollution is industrial or agricultural, it tends to find its way into the aquatic environment. Genotoxic pollutants can affect aquatic ecosystems directly, but their presence in water can also affect nonaquatic species via food chains or simply drinking water. Thus, it is necessary to develop and adjust strategies to enable both quantitative and qualitative analysis of the genotoxic effects of anthropogenic compounds and finally to integrate them into a battery of bioassays.

Among the numerous biomarkers of genotoxicity (DNA adducts, DNA strand breaks, chromosomal aberrations, sister chromatid exchange, micronuclei, etc.) used to evaluate DNA damage from exposure to environmental pollutants, the alkaline comet assay (CA) is particularly interesting. The 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 (strand breaks, alkali-labile, and excision repair sites) 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. Moreover, any nucleated cell can be used, and unlike other common cytogenetic assays such as the measurement of chromosomal aberrations, sister chromatid exchange, and micronucleus formation, cell division is not required.

The aim of the present study was to validate the CA using model compounds on the toad *Xenopus laevis,* for which ecotoxicological relevance is already well established, in a large array of studies focused on biomarkers such as induction of biotransformation enzyme activities (Bekaert, 1999; Gauthier et al., 2003), micronucleus (Ferrier et al., 1998) and DNA adduct formation (Bekaert et al., 2002), and teratogenic malformations (Chenon et al., 2002). Thus, extension of the end-point range in order to evaluate genotoxicity on the same biological model should provide an exhaustive assessment of the genotoxic impact in further biomonitoring studies.

The CA on *Xenopus laevis* larvae was carried out using well-known chemicals: the ethylating agent ethyl methanesulfonate (EMS), recognized as a potential carcinogenic compound, classified 2B in humans (IARC, 1987); the methylating agent methyl methanesulfonate (MMS); and the polycyclic aromatic hydrocarbon benzo[*a*]pyrene (B[*a*]P), considered a probable carcinogen, classified 2A in humans (IARC, 1999).

In this article we first propose to validate the choice of one of these chemicals for further use in ecotoxicological studies as a positive control because this is a preliminary requirement for a rigorous CA (Hartmann et al., 2003). Second, we report the comparison that we performed of the French Standard NF T90-325 micronucleus test (MNT; AFNOR, 2000) with the CA based on their ability to show damage in the DNA of *Xenopus laevis* erythrocytes exposed to these genotoxic agents. The MNT detects the induction of micronuclei, which are the consequence of chromosome fragmentation or malfunction of the mitotic apparatus; thus, clastogenic compounds and spindle poisons both lead to an increased number of micronucleated cells. For this last purpose, dose- and time-dependent responses were investigated and the sensitivity of the tests compared.

MATERIALS AND METHODS

Xenopus and Breeding

Sexually mature *Xenopus* were provided by the Developmental Biology Department of Paul Sabatier University (Toulouse, France). Males were injected with 400 IU of human chorionic gonadotropin (HCG), and females were injected with 700 IU of HCG. Males and females were placed together in normal tap water filtered through active charcoal at 22°C ± 2°C. Twelve hours later each pair was separated, and viable eggs were maintained in an aquarium also containing normal tap water filtered through active charcoal at 20°C–22°C until they reached a development stage appropriate for experimentation (usually 3 weeks). *Xenopus* larvae typically exhibited development stages 49–50 (Nieuwkoop and Faber, 1956) at the beginning of the experiments.

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. Exposure began on larvae that were at stage 50 of the *Xenopus* development table (Nieuwkoop and Faber, 1956). The larvae were taken from the same hatching in order to minimize

interanimal variability. The larvae were exposed in groups of 15 animals (100 mL/larvae) in 5-l glass flasks containing either the control medium—deionized tap water complemented with nutritive salts [294 mg/L $CaCl_2.2H_2O$, 123.25 mg/L $MgSO_4.7H_2O$, 64.75 mg/L $NaHCO_3$, 5.75 mg/L KCl (AFNOR, 1985)] or the test medium (EMS, MMS or B[*a*]P in deionized water complemented with nutritive salts). They were submitted to a 12 h light:12 h dark cycle. MMS and EMS were dissolved in water, whereas B[*a*]P was dissolved in dimethylsulfoxide (DMSO) at a final concentration of 0.05% (previously shown to be neither nongenotoxic nor toxic on *Xenopus*; data not shown) before being added to the water. The larvae were fed every day on dehydrated aquarium fish food. The flasks were partially immersed in water baths to maintain the water temperature at 22°C ± 0.5°C. The media in the control and exposed flasks were renewed daily. Acute toxicity to the treated larvae was analyzed visually (reduced size, diminished food intake, death) to ensure both genotoxicity tests were performed under nontoxic conditions. At the end of exposure, 12 days for the MNT and 1, 2, 4, 8, or 12 days for the CA, a blood sample was obtained from each anesthetized larva by cardiac puncture. Both tests were carried out on the same blood sample of each animal. The genotoxicity assessment was performed from the highest concentration not leading to any signs of acute intoxication of the exposed larvae.

Comet Assay (CA)

Experimental Procedure

Each experiment included a negative control, corresponding to larvae kept in control water. Alkaline CA (pH of the unwinding and electrophoresis buffer > 13) was performed essentially according to the procedure described by Singh et al. (1988) and finally adapted to *Xenopus laevis* larvae. Frosted microscope slides were cleaned and precoated with freshly prepared normal-melting-point agarose NMA (0.8% in phosphate-buffered saline, PBS), left at room temperature to allow the agarose to dry, and then kept refrigerated overnight at 4°C. After cardiac puncture an aliquot of heparinized blood cell suspension was immediately diluted 50-fold in PBS. Erythrocyte viability was routinely determined using the Trypan blue exclusion test (viability below 90% implies exclusion of the sample; Collins, 2002). Diluted blood was mixed with an equal volume of fresh low-melting agarose (LMA, 1%). Then 65 μL of this agarose cell suspension was spread on precoated slides and covered with a coverslip (22 × 50). Two slides were prepared per animal. After gelling for 7 min on an ice bed, the coverslip was gently removed. Then a third layer consisting of 90 μL of LMA (1% in PBS) was added and allowed to solidify for 7 min on ice before gently removing the coverslip. The slides were then immersed in a freshly prepared ice-cold lysis solution [4°C; 2.5M NaCl, 0.1M Na_2EDTA, 0.01M Tris, 1% Triton X-100, 10% DMSO (pH 10), set with NaOH] and kept for 1 h. Lysis and the next steps were performed in the dark under dim red light. The slides were then gently removed from the lysis solution and transferred into a horizontal electrophoresis tank containing cold, freshly prepared alkaline buffer [4°C; 0.3M NaOH, 1 mM Na_2EDTA (pH > 13)] for 20 min in order to let the DNA unwind. Electrophoresis was carried out in the same buffer for 20 min by applying an electric field of 20 V and adjusting the current to 300 mA. Finally, the slides were gently washed twice in a neutralization buffer (Tris-HCl 0.4M, pH 7.5) for 5 min before being dehydrated in absolute ethanol for 5 min. Slide analysis was performed using a confocal microscope (LSM 410 invert laser scan microscope; Zeiss) at 40× magnification after staining the slides (50 μL per slide of a 0.05 mM ethidium bromide solution). Quantitative assessment of DNA damage in erythrocyte nuclei was performed using Komet 4.1 image analysis software (Kinetic Imaging Ltd.), which computes the integrated intensity profile for each cell and estimates 34 parameters on each comet image. Thirty randomly selected cells were analyzed per slide. Comet images were randomly captured from the center of the slide, at a constant depth in the gel, avoiding the edges of the slide and overlapped figures. Comets with completely fragmented DNA (hedgehoglike figures with no apparent head) that could not be measured by the image analysis system were not taken into account. Data from two slides per animal and two animals per tested concentration of genotoxicant were pooled for the final processing. Two animals are preferred to the four or five included in each dose group at each sample time (one electrophoresis run), as recommended for conducting the *in vivo* alkaline comet assay (Hartmann et al., 2003), because of the known variability induced by two independent electrophoresis runs implied by the use of more than two animals per dose and three doses per substance for the same exposure time. To compare the genotoxic response resulting from the MNT and the CA, to evaluate genotoxicity with the CA at each sample time, and to evaluate a dose–response relationship, at least three doses are necessary for one exposure time.

CA Data Processing

Choice of Image Analysis Parameters

The question of a specific parameter choice in comet image analysis has not been completely solved. Preliminary studies (Mouchet, 2002) resulted in using at least two parameters because of the great variability in the representativeness of individual parameters among the 34 provided by the Komet 4.1 software according to the molecule tested, concentration in the medium, and/or exposure time. To compare different experiments, extent tail moment (ETM) and

tail length (TL) were selected because their correlation was the lowest among those parameters reported to be widely used by common consensus [ETM, olive tail moment (OTM), TL, and tail DNA (percentage of DNA in the tail)]. ETM computes the tail extent (TE) weighted by the tail DNA. TL represents the length of the tail (distance between the head and the last DNA fragment).

Statistical Analysis

Among the statistical tests found in the literature, two are particularly well adapted to the CA: the Mann–Whitney U test (MW) and the Kolmogorov–Smirnov test (KS; Vapnik, 1995). Following the results of a previous study (Mouchet, 2002), only the KS test was finally selected to be used in the present work. Its higher sensitivity makes it more suitable than the MW test because it is a distribution test, based on a comparison of two cumulative distribution functions of the samples tested and therefore uses the total information brought by the sample measurements, whereas the MW test reduces this information by computing rank sums.

Therefore, ETM and TL data (120 measurements per tested concentration) were analyzed using the KS test. For all tests, the chosen nondetection probability (NDP), α, that is, the probability of concluding the samples are statistically different when they are not, was 5%.

Definition of Evolution Coefficient (EC_{CA})

To quantify parameter evolution between two successive exposures to the same dose of the same agent, we propose to introduce the following evolution coefficient:

$$EC_{CA} = [[\{m(dose, time2) - m(dose, time1)\} - \{m(neg.control, time2) - m(neg.control, time1)\}]/\{m(dose, time1) - m(neg.control, time1)\}] \times 100 \quad (1)$$

where *m*(*dose, time1*) and *m*(*dose, time2*) and *m*(*neg.control, time1*) and *m(neg.control, time2)* are the means and corresponding negative controls, respectively, of the parameter for a given concentration of a genotoxicant at the two exposure times considered. The mean values of the parameter relative to the negative control are involved in the above expression in order to also take into consideration the evolution of the negative control.

The evolution percentage (EC_{CA}) can be positive (increased DNA damage between two successive exposure times) or negative (reduced DNA damage between two successive exposures).

Definition of an Induction Factor (IF_{CA})

To quantify the genotoxic potential of MMS and EMS on larvae for a given exposure versus the negative control and to compare their respective effects with the same dose and exposure time, we propose to introduce the following induction factor (IF_{CA}):

$$IF_{CA} = \{[m(dose, time1) - m(neg.control, time1)]/m(neg.control, time1)\} \times 100 \quad (2)$$

where *m*(*dose, time1*) is the mean value of the parameter computed for a given chemical agent and *m(neg.control, time 1)* is the mean value of the parameter relative to the negative control for the same exposure. The induction factor (IF_{CA}), expressed as a percentage, is positive if the mean value of the parameter induced by MMS or EMS exposure is greater than that of the negative control (increased DNA damage relative to the negative control) or negative if it is not (noted with a plus and a minus, respectively, in the tables).

Micronucleus Test (MNT)

Experimental Procedure

The *Xenopus* MNT was run according to the standard AFNOR NF T90-325 procedure (AFNOR, 2000). The negative control corresponds to untreated larvae, maintained in deionized water supplemented with mineral salts. The positive control corresponds to larvae exposed to 0.125 mg/L of B[*a*]P dissolved in DMSO and then added to the water. After 12 days of exposure, a single smear of blood was prepared per animal. 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 or more micronuclei was determined in a total sample of 3000 erythrocytes per larva.

Statistical Analysis

For each group of animals, the results [number of micronucleated erythrocytes per 1000 (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 Mac Gill et al. (1978) and consists of determining the theoretical medians of samples of size n (where $n > 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times IQR/\sqrt{n}$, where M is the median and IQR is the interquartile range. Under these conditions, the difference between the theoretical medians of the test groups and the theoretical median of the control group is significant to within 95% certainty if there is no overlap, and the result is then positive.

TABLE I. Results of *Xenopus* comet assay: Mean ETM and TL values in larvae exposed for 1–12 days to B[*a*]P

Exposure Time (days)	B[*a*]P Concentration	0 mg/L	0.125 mg/L	1 mg/L	10 mg/L
1	ETM	1.36 ± 0.66	1.68 ± 0.63↗	1.43 ± 0.38	0.56 ± 0.22↘
	TL	27.02 ± 5.3	28.07 ± 4.06↗	27.44 ± 2.97	19.12 ± 2.76↘
2	ETM	2.33 ± 0.72	1.62 ± 0.5↘	1.51 ± 0.55↗	1.53 ± 0.79↘
	TL	36.32 ± 7.26	26.13 ± 4.35↘	28.54 ± 4.11↘	24.83 ± 4.06↘
4	ETM	2.33 ± 0.64	1.29 ± 0.32↘	1.7 ± 0.49↘	0.78 ± 0.2↘
	TL	43.82 ± 6.2	30.8 ± 3.17↘	33.97 ± 3.2↘	20.65 ± 2.65↘
8	ETM	0.11 ± 0.03	0.14 ± 0.03	0.2 ± 0.04↗	0.13 ± 0.05
	TL	9.87 ± 1.2	10.53 ± 0.83	9.93 ± 0.86	9.45 ± 0.99
12	ETM	0.33 ± 0.09	0.27 ± 0.07↘	0.25 ± 0.05↘	0.3 ± 0.07
	TL	16.8 ± 1.58	13.7 ± 1.32↘	14.17 ± 1.21↘	15.33 ± 1.79↘

This table presents the calculated mean ETM (extent tail moment) and TL (tail length) values followed by their 95% confidence limits. The notations ↗ and ↘ indicate an increase or a decrease in DNA damage relative to the negative control, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$).

RESULTS

Acute Toxicity

No signs of toxicity were observed for *Xenopus* tadpoles exposed to B[*a*]P whatever the dose. Lethal intoxication was observed for tadpoles exposed to 10, 50, and 100 mg/L of MMS after the first day of exposure and to 100 mg/L of EMS between days 1 and 2, whereas no sign of toxicity was noted for those exposed to lower doses of either compound during the 12-day treatment.

Comet Assay

B[*a*]P Exposure

A genotoxic effect was observed only after the first day of exposure at 0.125 mg/L of B[*a*]P using the ETM and TL parameters and on day 8 at 1 mg/L of B[*a*]P using the ETM parameter (Table I). There was a significant reduction in DNA damage after 2, 4, and 12 days of exposure to 0.125 and 1 mg/L measured with both parameters and after 1, 2, 4, and 12 days of exposure to 10 mg/L with both parameters except with ETM after 12 days. Although the level of DNA damage increased or decreased according to duration of exposure time at a given concentration, the global tendency to have a significant reduction in the values of both parameters remained (except with ETM at 10 mg/L; Fig. 1) between days 1 and 12.

MMS Exposure

Two independent experiments were performed on *Xenopus* larvae exposed to MMS.

- *24-h Exposure:* The results showed a significant increase in DNA damage in larvae exposed to MMS measured with both parameters at the different doses compared to the negative control (Table II). A dose–response relationship was not observed.
- *2- to 12-Day Exposure:* The results showed that MMS exposure led to a significant increase in DNA damage at each dose and exposure time compared to the negative control measured with both parameters (Table III). An increasing dose–response relationship using both parameters was observable only until day 4 of exposure, followed by a decreasing relationship after 8 days and no relationship after 12 days.

Although evolution percentage showed positive or negative variations along the 12 days of exposure depending on the dose and the parameter considered, a significant decrease in DNA damage was observed between days 2 and 12 of the MMS exposure (ETM and TL; Table IV). Thus, the response of *Xenopus* larvae to 1, 3, and 6 mg/L of MMS has been globally shown to be inversely time dependent.

EMS Exposure

Two independent experiments were performed on *Xenopus* larvae exposed to EMS.

- *24-h Exposure:* EMS appeared to be genotoxic after a 1-day exposure at the highest concentrations (50 and 100 mg/L) when measured with both parameters (Table V). The results showed a significant reduction in DNA damage (ETM and TL) in larvae exposed to the lowest dose (10 mg/L).
- *2- to 12-Day Exposure:* Because of the lethal intoxication observed at 100 mg/L between days 1 and 2 of EMS exposure, we chose to investigate the dose and time response at the lowest doses tested (1, 3, and 6 mg/L), thus allowing comparison with the MNT and between both alkylating substances. The results showed a signifi-

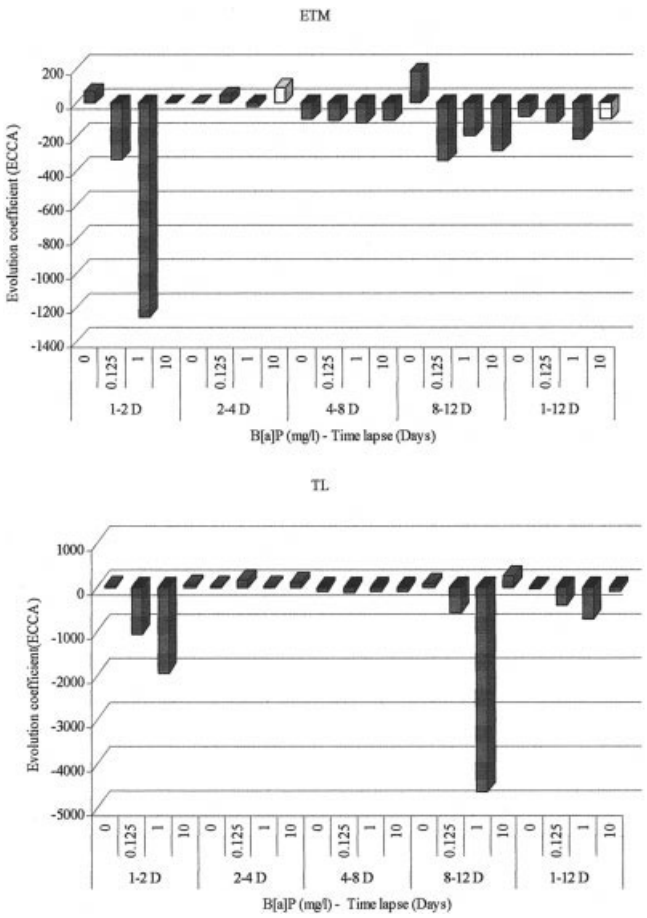

Fig. 1. Results of the *Xenopus* comet assay: evolution coefficients (EC_{CA}) of ETM and TL in larvae exposed from 1 to 12 days to B[*a*]P. These evolution coefficients were calculated as described in Eq 1. Therefore, a positive or a negative value corresponds to increased or decreased DNA damage, respectively, between two successive exposure times. Moreover, the dark graphs show cases for which a Kolmogorov–Smirnov test ($p = 0.05$) confirmed the difference in DNA damage (either increased or decreased) was statistically significant between two successive exposure times (all graphs are dark except the two graphs for the ETM parameter that correspond to exposures to 10 mg/L of B[*a*]P for 2–4 days and 1–12 days).

cant increase in DNA damage versus exposure time and EMS concentration except at the lowest dose (1 mg/L) on day 8 measured with the ETM parameter (Table III). An increasing dose–response relationship was noted at each exposure time except on day 4 (ETM).

Although percentage evolution showed positive or negative variations along the 12 days of exposure depending on the dose and the parameter, a significant decrease in DNA damage was observed at the highest dose (6 mg/L) between days 2 and 12 (Table IV). In contrast, DNA damage increased significantly in larvae exposed to the lowest dose of EMS (1 mg/L) during the same period (ETM and TL).

Micronucleus Test

Because the MNT has already been carried out many times in *Xenopus laevis* exposed to B[*a*]P under the same experimental conditions as those in our laboratory (Van Hummelen et al., 1989; Zoll-Moreux, 1991), we did not repeat this experiment in the present work. Van Hummelen et al. (1989) and Zoll-Moreux (1991) found B[*a*]P to be genotoxic at 0.06, 0.12, 0.25, and 0.5 mg/L. Figure 2 shows the number of MNE ‰ in larvae exposed for 12 days to MMS or EMS. A significant induction of MNE following a dose–response relationship relative to the control value was observed at all MMS concentrations up to 6 mg/L. However, a significant increase in MNE ‰ was observed only for EMS at the highest dose (50 mg/L).

DISCUSSION

Comet Assay

B[*a*]P and Alkylating Treatment

Exposure to B[*a*]P or to alkylating compounds in some cases led to decreased DNA damage in erythrocytes of *Xenopus laevis* by the end of the exposure compared to that in the control larvae. These results are in agreement with those of Devi et al. (2001), who observed a genotoxic response in the leukocytes of mice after exposure to $CdCl_2$ for 24 h, then a gradual decrease in DNA damage after 48, 72, and 96 h, suggesting DNA repair. Mouron et al. (2001) highlighted decreased DNA damage in human lung fibroblasts resulting from mechanisms of repair induced by metal exposure. Grover et al. (2001) observed a significant increase in the level of DNA damage in rat leukocytes after

TABLE II. Results of *Xenopus* comet assay: Mean ETM and TL values in larvae exposed to MMS for 24 h

	0 mg/L	1.56 mg/L	3.125 mg/L	6.25 mg/L
24 h				
ETM	1.54 ± 0.36	13.94 ± 2.28↗	12.58 ± 2.23↗	16.06 ± 1.42↗
TL	37.19 ± 2.71	66.5 ± 2.96↗	61.64 ± 3.04↗	74.99 ± 3.06↗

This table presents calculated mean values ETM (extent tail moment) and TL (tail length) values followed by their 95% confidence limits. The notation↗ indicates increased DNA damage relative to the negative control, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$).

TABLE III. Results of *Xenopus* comet assay: Mean ETM and TL values in larvae exposed for 2–12 days to MMS or EMS

	0 mg/L		1 mg/L		3 mg/L		6 mg/L	
	MMS	EMS	MMS	EMS	MMS	EMS	MMS	EMS
2-days								
ETM	0.67 ± 0.12	0.77 ± 0.14	17.29 ± 2.63↗	1.04 ± 0.23↗	21.47 ± 2.95↗	6.26 ± 1.22↗	22.73 ± 2.83↗	17.77 ± 2.96↗
TL	11.3 ± 0.63	20.13 ± 1.16	73.4 ± 2.08↗	22.33 ± 1.56↗	75.77 ± 2.82↗	55.67 ± 3.35↗	77.77 ± 2.77↗	68.67 ± 3.14↗
4-days								
ETM	0.58 ± 0.09	0.75 ± 0.13	10.11 ± 1↗	6.74 ± 1.42↗	11.99 ± 1.47↗	11.49 ± 1.85↗	19.36 ± 2.16↗	11.34 ± 1.65↗
TL	13.15 ± 0.72	18.2 ± 1.35	63.88 ± 2.9↗	59.31 ± 2.86↗	67.68 ± 2.88↗	65.87 ± 2.96↗	83.6 ± 3.34↗	67.04 ± 3↗
8-days								
ETM	0.37 ± 0.09	1.08 ± 0.22	14.27 ± 2.57↗	1.07 ± 0.15	13.20 ± 2.31↗	5.15 ± 0.80↗	9.98 ± 1.48↗	7.21 ± 1.42↗
TL	13.68 ± 1.18	22.22 ± 1.21	71.5 ± 3.71↗	24.58 ± 0.72↗	73.07 ± 3.24↗	51.73 ± 2.97↗	63.14 ± 2.63↗	57.89 ± 2.86↗
12-days								
ETM	0.42 ± 0.08	0.88 ± 0.17	10.98 ± 1.93↗	2.56 ± 0.46↗	7.49 ± 1.33↗	5.51 ± 1.69↗	11.75 ± 2.17↗	8.80 ± 1.73↗
TL	14.1 ± 0.7	20.41 ± 0.96	60.76 ± 2.83↗	30.84 ± 1.26↗	55.83 ± 2.88↗	54.97 ± 3.03↗	63.99 ± 2.99↗	59.05 ± 2.72↗

This table presents the calculated mean ETM (extent tail moment) and TL (tail length) values followed by their 95% confidence limits. The notation ↗ indicates increased DNA damage relative to the negative control, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$).

oral administration of $CdCl_2$ for 24 h, then a decrease after 48 h of exposure. In the present work, the reduction in DNA damage in the treated larvae could have stemmed from different cellular processes, including DNA repair activity. It has been well established that repair processes are genetically controlled (Moustacchi, 2000). Whatever kind of DNA damage occurs in *Xenopus*, a repair system can be induced that is either efficient or is saturated by the genotoxicant. This probably explains why the weakest dose of EMS led after 24 h of exposure to a reduction in DNA damage, whereas the highest doses led to increased DNA damage, suggesting saturation of repair activity at the high concentrations. Unlike with EMS exposure, after 24 h of B[*a*]P exposure, repair systems were not sufficiently induced at a low concentration (0.125 mg/L). Thus, a repair system seems to be induced under particular conditions: the concentration of the genotoxicant and/or the nature of the DNA damage and/or its location on the DNA molecule would condition its genetically controlled induction. Each repair system preferentially acts on a particular kind of damage (Friedberg et al., 1995). In our experiments the evolution of the DNA damage over the course of the exposure probably illustrates the constant balancing that occurs between the repair and damage processes. With B[*a*]P exposure, the decrease in DNA damage also could be a result of detoxication processes. Békaert (1999) showed that 0.15 mg/L B[*a*]P causes induction of EROD activity in *Xenopus* larvae from the second day of treatment. This is in agreement with the increase in DNA damage observed in the present work after a 1-day exposure to 0.125 mg/L of B[*a*]P, followed by a decrease after 48 h of exposure.

The CA results show that negative controls often exhibit significant background levels of DNA damage, which has been found to vary over time. Some studies (Singh et al., 1993; Guecheva et al., 2001) demonstrated that DNA strand breaks in negative controls could be induced by cell isolation. However, in our case, no prior cell isolation step was necessary because blood samples contain erythrocytes (1 lymphocyte per 1000 erythrocytes), which cannot explain the negative control background. As previously suggested by Wilson et al. (1998; in agreement with Pacifici and Davies, 1991), aerobic organisms are constantly exposed to endogenous and exogenous oxygen radicals and related oxidants, and although antiradical defense systems are present, a low level of oxygen radicals can induce oxidative stress in all cellular components, including DNA. Significant DNA damage in controls in some cell types may be a feature related to DNA packaging and background alkali-labile sites, rather than to exogenous strand breaks (Singh et al., 1989; Mitchelmore and Chipman, 1998). Moreover, as suggested by Liepelt et al. (1995), inconstant oxygen levels in test water lead to variation in DNA integrity. This background in negative controls can be considered unsurprising because a certain background level generally is observed in

TABLE IV. Results of *Xenopus* comet assay: Evolution coefficients (EC_{CA}) of ETM and TL after 2–12 days of MMS or EMS exposure

	1 mg/L		3 mg/L		6 mg/L	
	MMS	EMS	MMS	EMS	MMS	EMS
2–4 Days						
ETM	−42.69*	+2138.62*	−45.13*	+95.89*	−14.89*	−37.66*
TL	−18.30*	+1761.51*	−15.41*	+34.11*	+6*	+0.62*
4–8 Days						
ETM	+45.87*	−100.15*	+12.45	−62.08*	−48.80*	−42.10*
TL	+13.96*	−94.24*	+8.90*	−38.08*	−29.80*	−26.96*
8–12 Days						
ETM	−24.02*	−19528.00*	−44.90*	+13.64*	+17.83	+29.18
TL	−19.30*	+340.85*	−29.74*	+17.08	+0.88	+8.32
2–12 Days						
ETM	−36.48*	+528.80*	−66.00*	−15.59	−48.67*	−53.37*
TL	−24.87*	+372.46*	−35.28*	−2.77	−24.94*	−20.40*

These evolution coefficients are calculated as mentioned in Eq 1. Therefore, a positive or negative value corresponds to increased or decreased DNA damage, respectively, between two successive exposure times. An asterisk indicates that a Kolmogorov–Smirnov test ($p = 0.05$) confirmed the statistically significant difference in DNA damage (either increased or decreased) between two successive exposure times.

any biological test in negative controls, for example, in the micronucleus test.

Positive Control Investigation

B[*a*]P was not retained as a positive control because of the reduction of DNA damage observed in our exposure conditions. Strand breaks and alkali-labile sites, as the result of alkylating DNA bases (Tsuda et al., 2000), were easily measured with the CA in *Xenopus* and were those expected. Of the two alkylating agents, MMS was selected as a positive control in the CA on *Xenopus* because it has higher sensitivity than EMS (Fig. 3). In addition, MMS-induced damage, unlike that of EMS (and B[*a*]P), was measurable throughout the 12-day experiment. The greater sensitivity of MMS compared to EMS is supported by the work of La and Swenberg (1997), which showed that methylation reactions are 20 times more efficient than ethylation. Inconsistently, Singer (1982) reported that ethylating agents are more mutagenic because they have a greater affinity for oxygen affinity than do methylating compounds. That a reduction in DNA damage could be observed from the beginning to the end of the treatment for MMS in *Xenopus* is not a problem because the level of DNA damage remained significantly high at the end of treatment. The MMS concentration chosen (1.56 mg/L) is the lowest tested that induces significant levels of DNA damage as evaluated by both parameters. Moreover, this concentration induced levels of DNA damage, measured as the percentage of damaged cells (from 90% to 100%), similar to those induced by hydrogen peroxide, previously validated as a positive control for the *in vitro* CA in the same biological model (Mouchet, 2002).

The findings in the present work on the genotoxic effect of MMS, measured as a concentration, are in agreement with those described by Ralph et al. (1996) and Clements et al. (1997) for the amphibians *Rana clamitans* and *Rana catesbiana* exposed for 24 h to MMS and with those obtained by Mouchet (2002) in *Pleurodeles waltl* and by Deventer (1996) in zebra fish.

Micronucleus Assay

The positive response observed through the MNT after *Xenopus* exposure to 50 mg/L of EMS agrees with those described in the *in vivo* experiments carried out in the amphibian *Pleurodeles waltl* exposed to the same EMS dose (Jaylet et al., 1986). Moreover, the significant genotoxic effect measured in *Xenopus* erythrocytes after MMS exposure is consistent with those reported for the basal cells of rat skin (Nishikawa et al., 1999).

TABLE V. Results of *Xenopus* comet assay: Mean ETM and TL values in larvae exposed for 24 h to EMS

	0 mg/L	10 mg/L	50 mg/L	100 mg/L
24 h				
ETM	3.28 ± 1.11	3.20 ± 1.47↘	6.25 ± 1.36↗	9.65 ± 1.55↗
TL	47.4 ± 3.52	41.33 ± 3.74↘	62.88 ± 3.49↗	70.4 ± 3.84↗

This table presents the calculated mean ETM (extent tail moment) and TL (tail length) values followed by their 95% confidence limits. The notations ↗ and ↘ indicate increased or decreased DNA damage, respectively, relative to the negative control, confirmed by a Kolmogorov–Smirnov test ($p = 0.05$).

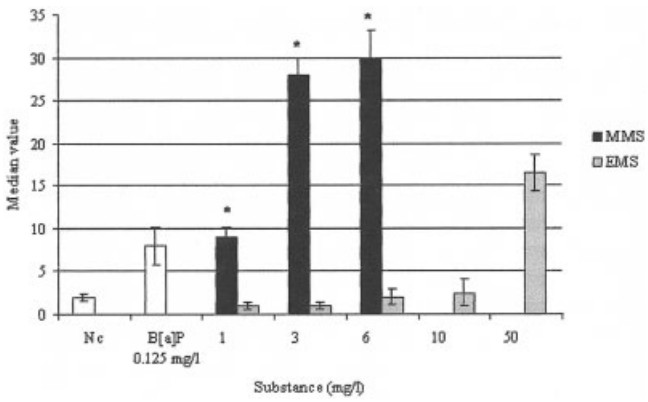

Fig. 2. Results of the *Xenopus* micronucleus assay: number of micronucleated erythrocytes (MNE) per 1000 cells in larvae exposed for 12 days to MMS or EMS. Concentrations of substances are expressed in milligrams per liter. Genotoxicity is expressed as the median number of MNE per 1000 (MNE ‰) with its 95% confidence limits (represented by vertical bars)—Nc, negative control group; B[*a*]P, benzo-[*a*]pyrene; *statistically significant difference ($p < 0.05$) from the control group (McGill et al., 1978). EMS concentrations of 10 and 50 mg/L are lethal to *Xenopus* larvae.

Comparison between CA and MNT

The *in vivo* MNT in amphibians detects chromosomal and/or genomic mutations (chromosomal damage and/or alteration of mitotic spindles), whereas the 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 incompletely repaired excision (sites present at the time of cell lysis). MMS and EMS exposure in *Xenopus* led to significant DNA damage measured by both the CA and the MNT, probably because of their clastogenic rather their aneugenic properties. This result suggests that MMS and EMS induced primary DNA lesions and that at least a fraction of them could give rise to some fixed mutations. Moreover, the genotoxic effects of MMS measured through the CA fit well with those observed in the MNT on a dose and exposure basis, suggesting a good relationship between both tests and assuming that single-strand breaks could induce chromosomal damage that resulted in micronucleus formation (Vodicka et al., 2001). This result is in agreement with that obtained by Deventer (1996) on the gill and liver cells of the zebra fish *Danio rerio* exposed to MMS. On the contrary, *Xenopus* larvae exposed to EMS exhibited a significant genotoxic response as assessed by the CA whatever the dose (1, 3, or 6 mg/L) after 2, 4, 8, and 12 days of exposure, whereas no positive response was obtained through the MNT after 12 days of exposure at the same doses.

In this way, the CA applied to *Xenopus* larvae for assessing genotoxicity seems to be a more sensitive method than the MNT. This might be attributed to the generation of repairable damage that does not persist after one mitotic cycle. So it is not surprising that low concentrations of genotoxicant can induce single-strand breaks, as revealed by the CA, without necessarily inducing micronucleus formation because of repair mechanisms. Previous experiments on B[*a*]P exposure showed significant induction of micronuclei using *Xenopus laevis* (Van Hummelen et al., 1989), suggesting that if B[*a*]P did not induce significant DNA damage in the present exposure conditions as assessed by the CA, it can nevertheless induce chromosomal and/or genomic mutations as revealed by the normalized MNT.

Genotoxic responses to both alkylating compounds can be measured from the first day of exposure with the CA, in comparison with the 12-day exposure required by the MNT, according to the standardized procedure (AFNOR, 2000). This is in agreement with the findings on zebra fish by Deventer (1996), who obtained genotoxic responses after an 8-h *in vivo* exposure of gill and liver cells using the CA versus a 6-day exposure using the MNT. However, the CA failed to show a genotoxic response in erythrocytes of *Xenopus* larvae exposed to B[*a*]P, contrary to the results found with the MNT. Taking into account such results and given their different end points and response times, both tests carried out in *Xenopus laevis* could be proposed in a complementary manner in a battery test system.

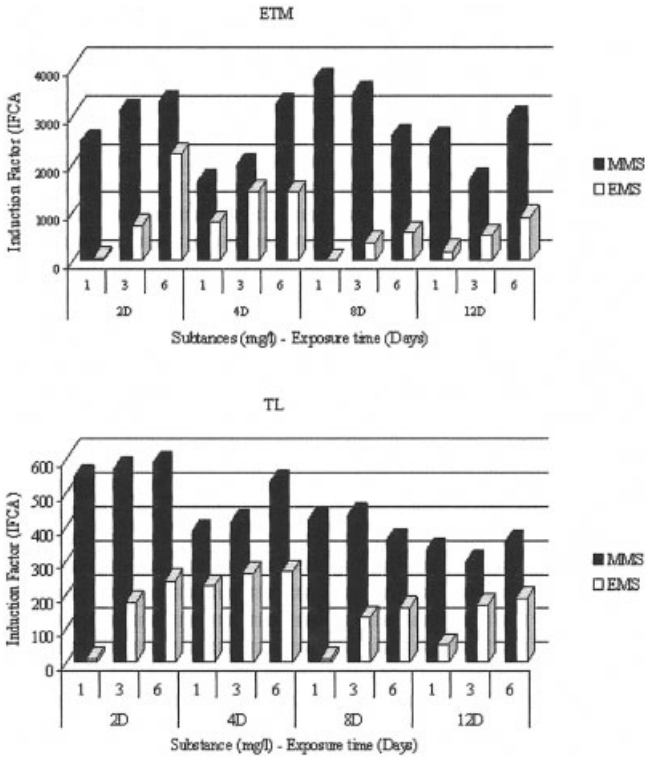

Fig. 3. Results of the *Xenopus* comet assay: induction factors (IF_{CA}) of ETM and TL in larvae exposed to MMS or EMS for 2–12 days. Each IF_{CA} was calculated with respect to the corresponding negative control, as explained in Eq 2.

CONCLUSIONS

The definition of a positive control for validation of the *in vivo* CA in *Xenopus* larvae is essential for further ecotoxicological investigations. The results of the present work led to choosing MMS among the substances studied. Moreover, the two alkylating agents studied, EMS and MMS (but not B[*a*]P), have been shown to increase micronucleus and DNA strand-break formation in *Xenopus laevis* tadpoles, whereas B[*a*]P was shown to induce only micronuclei under these conditions. Given that the induction of DNA damage measured in the two tests stems from different mechanisms and that they have different response times, the CA in *Xenopus* could be proposed as a relevant complementary method for a genotoxicity assessment of complex contaminated matrices and further biomonitoring studies.

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REFERENCES

AFNOR. 2000. NF T90-325. Qualité de l'eau—evaluation de la génotoxicité au moyen de larves d'amphibiens (*Xenopus laevis*, *Pleurodeles waltl*). 17 p.

Anderson D, Plewa MJ. 1998. The international comet assay workshop. Mutagenesis 13:67–73.

Békaert C. 1999. Impact écotoxicologique de sols pollués et de déchets sur le compartiment aquatique. Thèse de doctorat de l'Université Paul Sabatier de Toulouse. Centre de Biologie du Développement. 119 p.

Békaert C, Ferrier V, Marty J, Pfohl-Leszkowicz A, Bispo A, Jourdain MJ, Jauzein M, Lambolez-Michel L, Billard H. 2002. Evaluation of toxic and genotoxic potential of stabilized industrial waste and contaminated soils. Waste Manage 22:241–247.

Chenon P, Gauthier L, Loubières L, Séverac A, Delpoux M. 2002. Evaluation of the genotoxic and teratogenetic potential of a municipal sludge and sludge amended soil using the amphibian *Xenopus laevis* and the tobacco: *Nicotiana tabacum* L. var. *xanthi Dulieu*. Sci Total Environ 301:139–150.

Clements C, Ralph S, Petras, M. 1997. Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. Env Mol Mutagen 29:277–288.

Collins A. 2002. The comet assay. Principles, applications, and limitations. Methods Mol Biol 203:163–177.

Deventer K. 1996. Detection of genotoxic effects on cells of liver and gills of *B. rerio* by means of single cell gel electrophoresis. Bull Environ Contam Toxicol 56:911–918.

Devi KD, Banu BS, Mahboob M, Jamil K, Grover P. 2001. *In vivo* genotoxic effect of cadmium chloride in mice leucocytes using comet assay. Teratog Carcinog Mutagen 21:325–333.

Fairbairn DW, Olive PL, O'Neill KL. 1995. The comet assay: a comprehensive review. Mutat Res 339:37–59.

Ferrier V, Gauthier L, Zoll–Moreux C, L'Haridon J. 1998. Genotoxicity tests in amphibians—a review. Microscale testing in aquatic toxicology: advances, techniques and practice. Environ Toxicol 35:507–519.

Friedberg G, Errol C, Walker C, Siede W. 1995. DNA repair and mutagenesis. Washington, DC: American Society for Microbiology Press. 698 p.

Gauthier L, Tardy E, Mouchet F, Marty J. 2003. Biomonitoring of the genotoxic potential (*micronucleus assay)* and detoxifying activity (EROD induction) in the River Dadou-France, using the amphibian *Xenopus laevis.* Sci Tot Environ 323:47–61.

Guecheva T, Henriques JAP, Erdtmann B. 2001. Genotoxic effects of copper sulphate in freshwater planarian *in vivo*, studied with the single-cell gel test (comet assay). Mutat Res 497:19–27.

Grover P, Banu BS, Devi KD, Begum S. 2001. *In vivo* genotoxicity effects of mercuric chloride in rat peripheral blood leucocytes using comet assay. Toxicol 167:191–197.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. 2003. Recommendations for conducting the *in vivo* alkaline comet assay. Mutagenesis 18:45–51.

IARC. 1987. Overall evaluations of carcinogenicity: an updating of IARC Monographs Volumes 1 to 42. In: IARC monographs on the evaluation of carcinogenic risks to humans and their supplements. Lists of evaluations. IARC Supplement No. 7, Lyon, France. 440 p.

IARC. 1999. Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide. In: IARC Monographs on the evaluation of carcinogenic risks to humans and their supplements. Lists of evaluations. IARC Volume 71, Lyon, France. 1589 p.

Jaylet A, Deparis P, Ferrier V, Grinfeld S, Siboulet R. 1986. A new micronucleus test using peripheral blood erythrocytes of the newt *Pleurodeles waltl* to detect mutagen in fresh water pollution. Mutat Res 164:245–257.

La KD, Swenberg JA. 1997. Carcinogenic alkylating agents. Comp Toxicol 12:111–140.

Liepelt A, Karbe L, Westendorf J. 1995. Induction of DNA strand breaks in rainbow trout *Oncorhynchus mykiss* under hypoxic and hyperoxic conditions. Aquat Toxicol 33:177–181.

McGill R, Tuckey J, Larsen W. 1978. Variations of box plots. Am Statist 32:12–16.

McKelvey VJ, Green MHL, Schmezer BL, Pool-Zobel BL, De Meo MP, Collins A. 1993. The single cell gel electrophoresis assay (comet assay): a European review. Mutat Res 288:47–63.

Mitchelmore CL, Chipman JK. 1998. DNA strand breakage in aquatic organisms and the potential values of the comet assay in environmental monitoring. Mutat Res 339:135–147.

Mouchet F. 2002. Validation du test comète sur larves d'amphibiens (*Xenopus laevis* et *Pleurodeles waltl*) et application à l'évaluation du potentiel génotoxique de sols, sédiments et déchets contaminés—comparaison avec le test micronoyau amphibian [PhD thesis]. Université Paul Sabatier, Toulouse, France. 306 p.

Mouron SA, Golijow CD, Dulout FN. 2001. DNA damage by cadmium and arsenic salts assessed by the single cell gel electrophoresis assay. Mutat Res 498:47–55.

Moustacchi E. 2000. DNA damage and repair: consequences on dose-responses. Mutat Res 464:35–40.

Nieuwkoop PD, Faber J. 1956. Normal tables of *Xenopus laevis* (Daudin). Amsterdam, The Netherlands: North-Holland Publishing.

Nishikawa T, Haresaku M, Adachi K, Masuda M, Hayashi M. 1999. Study of a rat skin *in vivo* micronucleus test: data generated by mitomycin C and methyl methanesulfonate. Mutat Res 444:159–166.

Olive PL, Banath JP, Durand RE. 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat Res 122:86–94.

Östling O, Johanson KJ. 1984. Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123:291–298.

Pacifici RE, Davies KJA. 1991. Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of ageing revisited. Gerontology 37:166–180.

Ralph S, Petras M, Pandrangi R, Vrzoc M. 1996. Alkaline single cell gel (comet) assay and genotoxicity monitoring using two species of tadpoles. Environ Mol Mutagen 28:112–120.

Rojas E, Lopez MC, Valverde M. 1999. Single cell gel electrophoresis assay: methodology and applications. J Chromatogr B Biomed Applied 722:225–254.

Singer B. 1982. Mutagenesis from a chemical perspective: nucleic acid reactions, repair, translation, and transcription. In: Lemont JF, Generoso WM, editors. Molecular and cellular mechanism of mutagenesis. New York: Plenum. p 1–41.

Singh NP, McCoy MT, Tice RR, Schneider LE. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191.

Singh NP, Danner DB, Tice RR, McGregor MT, Collins GD, Scheider EL. 1989. Abundant alkali sensitive sites in DNA of human and mouse sperm. Exp Cell Res 184:461–470.

Singh NP, Tice RR, Stephens RE, Schneider LE. 1993. A microgel electrophoresis technique for the direct quantitation of DNA damage and repair in individual fibroblasts cultured on microscope slides. Mutat Res 252:289–296.

Speit G, Hartmann A. 1999. The comet assay (single-cell gel test)—a sensitive genotoxicity test for the detection of DNA damage and repair. In: Henderson DS, editor. Methods in molecular biology. Volume 113, DNA repair protocols: eucaryotic systems. Totowa, NJ: Humana Press. p 203–212.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E. 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 35:206–221.

Tsuda S, Matsusaka N, Madarame H, Miyamae Y, Ishida K, Satoh M, Sekihashi K, Sasaki YF. 2000. The alkaline single cell electrophoresis assay with eight mouse organs: results with 22 mono-functional alkylating agents (including 9 dialkyl *N*-nitrosoamines) and 10 DNA crosslinkers. Mutat Res 467:83–98.

Van Hummelen P, Zoll, C, Paulussen J, Kirsch-Volders M, Jaylet A. 1989. The micronucleus test in *Xenopus*: a new and simple *in vivo* technique for detection of mutagens in fresh water. Mutagen 4:12–16.

Vapnik VN. 1995. The nature of statistical learning theory. New York: Springer Ed.

Vodicka P, Koskinen M, Vodicka L, Stetina R, Smerak P, Barta I, Hemminki K. 2001. DNA adducts, strand breaks and micronuclei in mice exposed to styrene by inhalation. Chemico-Biol Interac 137:213–227.

Wilson JT, Pascoe PL, Parry JM, Dixon DR. 1988. Evaluation of the comet assay as a method for the detection of DNA damage in the cells of a marine invertebrate, *Mytilus edulis L.* (Mollusca: Pelecypoda). Mutat Res 399:87–95.

Zoll-Moreux C. 1991. Conséquences de la contamination du milieu hydrique par des sels de mercure, du benzo(a)pyrène ou des pesticides organochlorés, chez deux amphibiens *Pleurodeles waltl* et *Xenopus laevis* [PhD thesis]. Université Paul Sabatier, Toulouse, France. 204 p.