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Biomonitoring of the genotoxic potential (micronucleus assay) and detoxifying activity (EROD induction) in the River Dadou (France), using the amphibian *Xenopus laevis*

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Abstract

Within the framework of a general survey of the water quality of the river Dadou (Tarn, France), different physico-chemical parameters were measured and an inventory of the fish population was made along the water course, around the Rassisse dam. With the aim of monitoring the potential genotoxic effects and the detoxifying activities induced in organisms exposed to the river water, two in vivo bioassays were performed in laboratory experiments, using larvae of the amphibian *Xenopus laevis*. The first was the micronucleus test, using red blood cells, and the second the assay of ethoxyresorufin-*O*-deethylase (EROD) induction in the liver of exposed animals. Eight water samples were taken from the river and at outlet points from the two major industrial activities of the studied section of the water course: a spar-fluor mine and a water treatment plant. Genotoxic impact and EROD induction were measured in the larvae. The effluent of the filter-washing process from the water treatment plant was found to be particularly genotoxic, even after dilution in pure reconstituted water, but no particular genotoxicity was found, either in Dadou river water, or in the effluents from the mine. On the other hand, most of the water samples tested produced a clear induction of EROD activity compared to the level of enzymatic activity found in the liver of larvae reared in the river water sampled upstream of the industrial activities. These results were interpreted taking into account (i) the high concentrations of pollutants (fluorine and manganese) measured in the river water, (ii) the very low population levels inventoried in the downstream section of the river and (iii) the possible interactions between the substances present in the river water, particularly the classical EROD inducers PAHs and PCBs.

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1. Introduction

Increased environmental pollution can be attributed to a variety of factors resulting from new industrial and agricultural technologies, together with changes in our way of life. In addition, the nature of the pollution itself has become more diverse. Regardless of its origin, pollution tends to find its way into the aquatic environment. Genotoxic pollutants affect the aquatic ecosystems and their presence in water can also have repercussions on non-aquatic species, via food chains, or simply as a result of drinking the water. One should therefore be aware of the hidden risks stemming from potential genotoxic substances in the aquatic environment. Moreover, a considerable time may elapse between the action of the mutagenic agent and the outward signs of its effects. The relationship between cause and effect may thus become obscure.

The mutagenic risk is particularly apparent in prokaryotes, and is readily discernible in plants and animals with a rapid rate of reproduction, although it is often not very perceptible in plants and animals (including humans) with a slower reproduction rate. The mutagenicity of an unknown substance is usually evaluated by placing it in contact with a living system, which is then examined for genetic damage. A number of tests have been developed, using either plants or aquatic animals (see the reviews of Jaylet et al., 1990; Jaylet and Zoll, 1990; Zoll et al., 1990; Godet et al., 1993), which can potentially be used to assess the genotoxic potency of water samples. The tests 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 biomarkers and a sensitive model for environmental studies. Larvae can be reared, not only in containers filled with unconcentrated water samples (laboratory conditions) but also in running water of various sources (factory effluents, surface water, river waters, …).

We decided to use the amphibian *Xenopus laevis* to monitor the long-term effects of exposure to river water. Two genotoxic endpoints were analysed in larvae of these animals: the induction of micronucleated erythrocytes in the circulating blood and the induction of 7-ethoxyresorufin-*O*-deethylase (EROD) activities in the liver.

In amphibian larvae, as in most eukaryotes, genome mutations may result in the formation of micronuclei, which is the consequence of chromosome fragmentation or malfunction of the mitotic apparatus. Thus, clastogenic compounds and spindle poisons both lead to an increase in the number of micronucleated cells. Induction of micronuclei has been widely used for genotoxicity testing. In aquatic vertebrates, the micronucleus test has been carried out on different species of fish (Das and Nanda, 1986; Metcalfe, 1988; Carrasco et al., 1990). Amphibians have also proved to be valuable biological models for micronucleus induction (Jaylet et al., 1986; Krauter et al., 1987).

In aquatic organisms, EROD activities have been measured mainly in fishes, both marine (Burgeot et al., 1994) and continental species (Vindimian et al., 1993; Anderson et al., 1995; Collier et al., 1995). Cytochrome P4501A synthesis is mainly induced in aquatic organisms exposed to chemical contaminants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins. Many authors have described significant induction of cytochrome P4501A correlated with various types of pollution in fresh water, mainly in fishes (Vindimian and Garric, 1989; Masfaraud et al., 1990). Of the different monooxygenase activities, EROD activities were found to be the most sensitive to monitors of pollution in the aquatic environment (Monod et al., 1988). Relatively few studies have reported the use of amphibians to monitor the induction of cytochrome P4501A activities in response to environmental pollution. Aryl hydrocarbon hydroxylase activities were measured in *Ambystoma tigrinum* (Busbee et al., 1975) and *Pleurodeles waltl* (Marty et al., 1989). Hepatic microsomal mixed-function oxidases were studied in anuran species: *Rana temporaria* (Harri, 1980), *Rana catesbeiana*, *Rana nigromaculata*, *Bufo bufo japonicus* and *Xenopus laevis* (Noshiro and Omura, 1984). In our laboratory, Bekaert (1999) measured EROD activity in the liver of *xenopus* larvae exposed to benzo(*a*)pyrene and she extended the application of
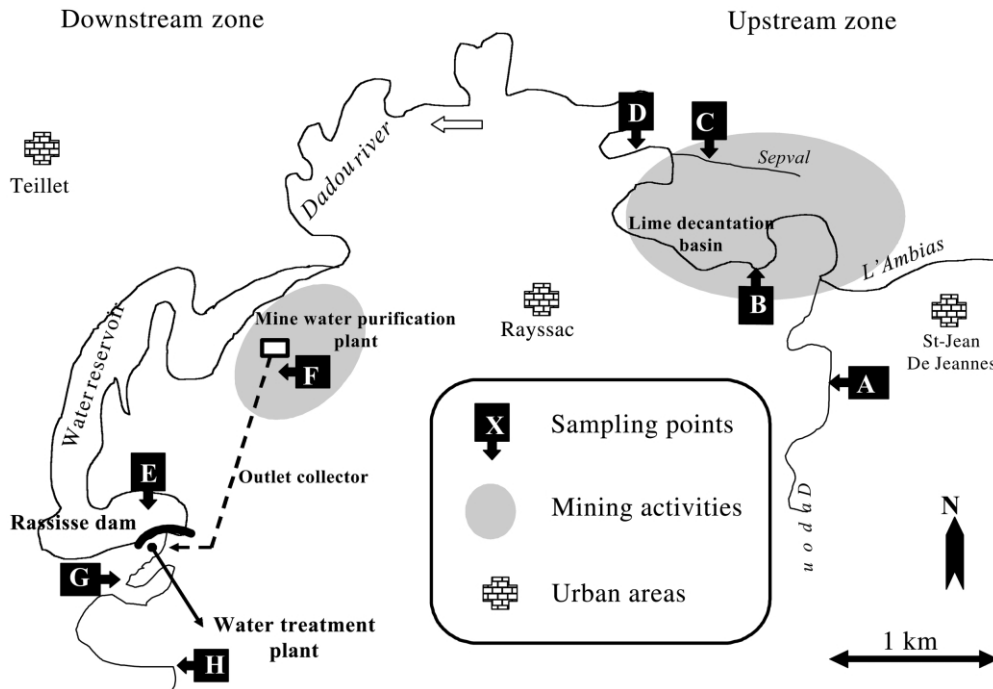
Fig. 1. Schematic representation of the Dadou river in the studied section showing the principal outlets of the surrounding industrial activities and the situation of the different sampling points (A to H). Dotted lines: collectors.

this bio-assay to study contaminated environmental mixtures.

In the framework of this study, we used two biomarkers (micronucleus formation and EROD activity induction) in the same organism to control the water quality of River Dadou, France (Tarn) around the dam of Rassisse. This work was part of a general survey of the river performed after the observation of a high mortality level in the natural fish population along this portion of the watercourse, including the reservoir of the Rassisse dam.

2. Materials and methods

2.1. General situation and water sampling

The area studied here is a rural region, where two major industrial activities are present: a fluor-spar mine and a water treatment plant. These activities directly affect the River Dadou through the outlets from the purification plants of the mine and from the water treatment plant (Fig. 1).

Eight water samples were taken directly from the river or its tributaries and from outlet points of the industrial activities. The sampling sites were labelled in alphabetical order from upstream to downstream of the river and are described below.

Point A was the most upstream sampling point in the River Dadou. This point, far from any industrial contamination, was considered as the upstream control of the experiment. At point B (1500 m downstream of point A), water samples were taken in a small stream tributary of the river, at the outlet of the decantation basin of the plant that treats the water extracted from the mine. This effluent is characteristic of the main outlet of the fluor-spar mining activity. Sampling point C reflects the contribution of a secondary waste activity of the mine, which is the production of deads. Water samples were taken from a second small stream, tributary of the River Dadou, that

collects running water through the deads of the mine. At point D, water was sampled in the river, downstream of points A (4 km), B and C, and upstream of the Rassisse reservoir. This point enabled the global impact of the mining activity on the river to be analysed. Sampling point E is situated in the Rassisse reservoir and corresponds to the intake of the water treatment plant ($\sim$14 km downstream of point A). At point F, water was sampled at the outlet of the general purification plant of the mine. A collector carries the effluent to the river, downstream of the Rassisse dam. Water samples were also taken at point G, at the outlet of the water treatment plant ($\sim$400 m downstream of point E). The effluent here corresponds to the water from the washing of the sand filters of the plant. A collector brings the effluent to the river. Point H—located approximately 16 km downstream of point A—was the most downstream site of the River Dadou sampled in this work.

For each water sample, 40 l was collected in 10-l plastic containers for subsequent laboratory tests with amphibian larvae. Water samples were maintained at 4 °C in the dark before testing. Physical parameters (pH and dissolved oxygen) were measured in water samples using a pH 96 WTW pH meter and an oxi 96 WTW oxy meter. These two properties were followed throughout the exposure of the animals to the water. Electrical fishing was also performed to evaluate the population level in the river at sampling sites A, D and H. Physico-chemical analyses were also performed at the water sampling sites. These included pH, conductivity (measured at 20 °C), redox potential, TH (°F), Al, Cu, Mn and Fl levels.

2.2. The amphibian micronucleus assay using Xenopus larvae

Originally, the test procedure was established in our laboratory on the anuran *Xenopus laevis* (the South African clawed frog or common platanna; family Pipidae) by Van Hummelen et al. (1989) and Zoll et al. (1990). The test is particularly suitable for evaluating promutagens and genotoxic agents with a direct action, as well as complex environmental mixtures, such as urban and indus-

trial effluents (Zoll-Moreux, 1991; Zoll-Moreux and Ferrier, 1999). Furthermore, their abundant egg-laying, the availability of larvae throughout the year and their consumption of standardized dehydrated food confer certain advantages on *Xenopus* over the urodeles (Gauthier, 1996).

The test procedure used in this study was described in the French Standard NF T 90-325 (AFNOR, 2000), which is the French National Organization for quality regulations. Briefly, it consists in the evaluation of the number of micronucleated red blood cells (RBCs) in larvae exposed for 12 days at 22 ± 0.5 °C under a normal light–dark cycle. The larvae are exposed by groups of 20 animals in 5-l glass flasks containing 2 l of water (100 ml per larva). The test begins on larvae at stage 50 of the development table of *Xenopus* (Nieuwkoop and Faber, 1956). Throughout the period of exposure, water and food are renewed daily. Positive and negative controls were included in each experiment described here. The negative control was filtered over sand and active charcoal, supplemented with mineral salts. The positive control was a 20 mg/l solution of cyclophosphamide monohydrate. After exposure, blood samples were taken from each larva by cardiac puncture and a single smear was prepared per animal. After fixing in methanol and staining with hematoxylin, the smears were screened under the microscope (oil immersion lens, $\times$1500). The number of RBCs that contained one or more micronuclei was determined in a total sample of 1000 erythrocytes per larva. For each animal, the mitotic index (number of mitotic erythrocytes per 1000) was evaluated by scoring the dividing cells of the circulating blood on the smears. For each group of animals, the results (number of micronucleated RBCs per 1000) 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 in determining the theoretical medians of samples of size n (where $n \geqslant 7$) and their 95% confidence limits expressed by $M \pm 1.57 \times IQR/\sqrt{n}$, where M is the median and IQR is the Inter-Quartile Range. 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. The result is then positive.

2.3. EROD activities in the larvae of the amphibian Xenopus laevis

After exposure of the *Xenopus* larvae for 12 consecutive days to the water to be tested and after cardiac puncture, the livers of the larvae were taken. After removal of the gall bladder, the livers of each treated group were pooled. They were then homogenized with a potter (1200 rpm) in the homogenization buffer (phosphate Na/K, 50 mM; KCl, 1.15 %; PMSF, 1 mM; Aprotinine, 14 U/ml).

Protein was assayed in homogenate samples by the Bradford method (Bradford, 1976), using a bovine serum albumin solution (BSA Sigma) as standard.

The EROD activity of the homogenate was measured in an incubation buffer (phosphate buffer, 100 mM; NADP, 0.5 mM; G6P, 5 mM). Before protein assay, 10 U of G6PDH (Boehringer) was added to 10 ml of the buffer. The incubation conditions were as follows: incubation buffer (500 μl), water (240 μl), liver homogenate (250 μl), substrate, 7-ethoxyresorufin (10 μl). After incubation for 2 min at 20 °C, the reaction was stopped by adding 2 ml of acetone. The resorufin fluorescence emitted during the incubation period was measured using a Jobin-Yvon spectrofluorimeter on a 2-ml sample after centrifugation (5 min at 10 000 rpm). The quantity of resorufin in the samples was evaluated by comparison with a pre-established standard curve.

EROD activities are expressed as pmoles of resorufin formed per minute per milligram of protein in the liver homogenate and per milligram of fresh liver. The level of induction is expressed as the ratio of the activity of treated larvae/activity of control larvae.

In order to analyse more fully the results obtained, *Xenopus* larvae were subjected to four concentrations (4, 40, 100 and 200 ppb) of a PAH, benzo(*a*)pyrene (B[*a*]P), and EROD activity and micronucleus formation were analysed. In this way,

it was possible to compare the EROD activities measured in the river water with those inducted by known concentrations of B(*a*)P in the rearing medium of the animals and to compare it with the induction level of micronucleated RBCs in the exposed larvae. In these experiments, B(*a*)P was dissolved in dimethyl sulfoxide (DMSO) before addition to the water to be tested. A solvent control containing the same concentration of DMSO (0.1%) was tested in parallel.

3. Results

The physical parameters (pH, dissolved oxygen and temperature) measured in the water samples before testing on amphibian larvae showed that the characteristics of the effluents were compatible with normal rearing of the amphibians under the conditions of the test ($6 < pH < 9$; dissolved $O_2 \geqslant 60\%$; temperature from 21.5 to 22.5 °C), except for the water sampled at point C, which had a very low pH (3.95). This acidic effluent required neutralization before the amphibian larvae were exposed to it.

Before genotoxicity testing, preliminary toxicity assays were performed (10 *Xenopus* larvae per liter of water) to evaluate the feasibility of the test under non-lethal conditions. We observed no particular toxicity to the larvae reared for 6 days in water samples A, D, E and H. On the other hand, all the *Xenopus* larvae reared in water samples B, C, F and G displayed consequences of severe intoxication (reduced size, asphyxia, diminished food intake, death); for these effluents, preliminary dilutions with pure water were required before testing for micronucleus induction over a period of 12 days.

3.1. The micronucleus assay using Xenopus larvae

The results obtained with diluted and undiluted effluents are presented in Table 1 and Fig. 2. The water sampled at points A, E and H, corresponding to the water taken far from any industrial outlet points, gave clearly negative responses. The larvae reared in water samples taken from the points B, C, D and F produced more micronuclei than the negative control, although the increase was not

Table 1
Results of the *Xenopus* micronucleus assay

Water sample Concentration (ml/l)	Negative control	Positive control	A 1000	B 500	B 250	C 1000	C 500	D 1000	E 1000	F 250	G 1000	G 500	H 1000
Lower extreme	0	5	0	0	1	0	1	0	0	1	0	2	0
Lower quartile	0	5.5	0	2	1	1.5	1	1	1	1.5	1.5	3	0
Median	1	8	1	2	2	2	2	2	1	2	3	4.5	1
Confidence limit	(0.19–1.81)	(5.57–10.43)	(0.215–0.785)	(1.63–2.37)	(1.215–2.785)	(1.41–2.59)	(1.215–2.785)	(1.3–2.7)	(0.62–1.38)	(1.46–2.54)	(1.87–4.13)	(2.52–6.48)	(0.215–1.785)
Upper quartile	2	11.5	2	3	3	2.5	3	3	2	3	4	7	2
Upper extreme	4	14	2	4	5	3	9	4	3	4	6	11	6
Mean	1.46	8.66	1.13	2.27	2.13	1.86	2.69	1.95	1.35	2.32	2.75	5.3	1.56
Results		+	−	−	−	−	−	−	−	−	+	+	−
Number of animals	15	15	16	18	16	7	16	20	17	19	12	10	16

Frequencies of micronucleated erythrocytes (per 1000 cells) in larvae reared for 12 days in water samples. Genotoxicity is expressed as the values of the medians (number of micronucleated erythrocytes per thousand) and their 95% confidence limits. −, negative result. +, positive result.
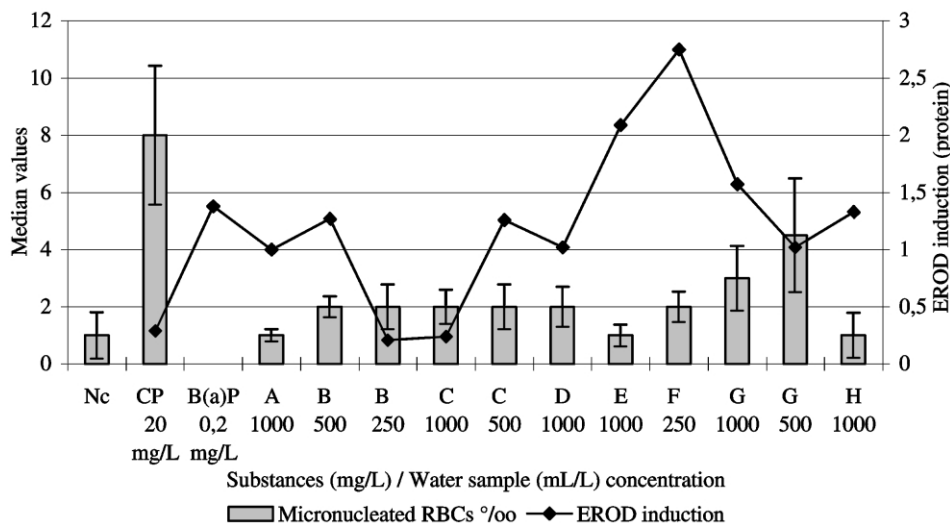
Fig. 2. Results of the *Xenopus* micronucleus assay (frequencies of micronucleated erythrocytes—per 1000 cells—in larvae reared for 12 days in water samples from the River Dadou) and of the EROD activities (EROD induction protein) measured in their liver.

statistically significant. In these cases, the test response was considered to be negative. All these samples were effluents from mining activities. It should be noted that because of the highly toxic effects previously detected with effluents B, C and F, the highest effluent concentrations tested with the corresponding water samples were, respectively, 500, 1000 and 250 ml/l. In the case of effluent C, the micronucleus assay was only performed on the seven larvae that survived after 12 days of exposure to the undiluted water samples. Larvae reared in effluent F diluted to 250 ml/l survived and consequently the micronucleus assay could be performed on all of them. Water sample G induced a significant increase of micronucleated RBCs in the larvae exposed to the raw and to the diluted effluents (500 ml/l). This latter water sample led to the highest induction of micronucleated erythrocytes observed in our experiments.

3.2. EROD activities in the larvae of the amphibian Xenopus laevis

The measured EROD activities are expressed both in terms of the liver homogenate protein concentration and of the whole liver (Table 2). They represent the quantity (pmole) of resorufine produced per minute in the incubation medium. We observed a substantial fluctuation in the weights of the liver taken from the treated larvae. The EROD activities expressed in terms of the fresh weights of the livers take into account the physiological condition of the larvae, compared to the classical enzymatic activity expressed per milligram protein. In both cases, the results revealed the same overall variations and appeared coherent. Compared to those of the larvae exposed to the other water samples, the liver homogenates of the amphibians reared in the water from site C contained significantly lower EROD activity. Moreover, the larvae reared for 12 days in effluent C diluted twofold (500 ml/l) expressed higher enzymatic activities than the larvae exposed to the undiluted effluent. The animals reared in the water from sample C had very small, hemolysed livers. These physiological problems probably contributed to the observed alterations of the enzymatic activities in the livers of these animals. Liver fresh weights of larvae reared in diluted effluent C were normal compared to those of larvae raised in the undiluted effluent (Table 2); accordingly, only the enzymatic activities measured in the diluted effluent (500 ml/l) were considered for interpretation.

Table 2
EROD activities measured in the liver of larvae exposed to water samples from the River Dadou

| Substance/water sample | CP | B(a)P | A | B | B | C | C | D | E | F | G | G | H |
Concentration	20 mg/l	0.2 mg/l	1000	500	250	1000	500	1000	1000	250	1000	500	1000
Liver weight (mg)	87.8	56.5	49.2	64.8	55.2	14.7	43.2	49.1	65.5	21.4	24.5	23.6	41.1
EROD activity (pm/min/mg liver)	0.83	3.02	1.93	1.22	0.61	0.52	3.46	1.12	7.84	9.29	4.84	4.07	6.82
EROD induction (liver)	0.43	1.56	1	0.63	0.3	0.27	1.79	0.58	4.1	4.8	2.51	2.11	3.53
Protein concentration (mg/ml homogenate)	3	2.2	3.5	1.7	5.2	1.9	4.9	2	6.7	3	2.7	3.6	9.1
EROD activity (pm/min/mg protein)	16.1	77.5	56.1	71.4	11.7	13.6	70.7	57.2	117.4	154.3	88.3	57.2	75.1
EROD induction (protein)	0.29	1.38	1	1.27	0.21	0.24	1.26	1.02	2.09	2.75	1.57	1.02	1.33

The concentration of the water samples is expressed in ml/l. Liver weight represents the total weight of the livers measured in groups. Protein concentration is expressed as milligram protein per milliliter liver homogenate. EROD induction is expressed with respect to fresh weight of liver (liver) and with respect to the protein in the liver homogenates (protein). Upstream sample A was the negative control for the experiment. CP, cyclophosphamide monohydrate. B(a)P, benzo(a)pyrene.

Compared to the basic EROD activity measured in the larvae exposed to water sampled at the most upstream point (A), the highest induction levels were found in larvae reared in water from the outlet of the purification plant of the mine (Fig. 2), even after dilution (250 ml/l). Very high inductions were obtained with the water sampled from the Rassisse reservoir (E). Water samples from the most downstream point of the river (H) also strongly induced the enzyme (×3.5), particularly when the activity was expressed in terms of liver weight. We observed a modest induction of EROD in larvae reared in water from the outlet of the water treatment plant (G) and in running water from the deads of the mine (C) after dilution. Water samples taken at the outlet of the decantation basin of the mine (B) and water sampled upstream of the dam in the Dadou river (D) did not lead to enzymatic inductions in our experiments.

The results obtained with the two positive controls demonstrate that B(a)P at 0.2 mg/l is an inducer of EROD activity in the liver of *Xenopus* larvae; in contrast, cyclophosphamide at 20 mg/l, which is the classical positive control used in the amphibian micronucleus assay, is not.

3.3. Comparison of EROD induction and micronucleated erythrocyte formation in Xenopus larvae exposed to known B(a)P concentrations

The results of this experiment are reported in Table 3. After exposure to the four B(a)P concentrations tested (4, 40, 100 and 200 ppb), the treated larvae and the controls were analysed for micronucleus formation and EROD activity. The results demonstrate that DMSO did not induce EROD activity in the liver of the larvae and did not lead to the formation of micronuclei in RBCs of *Xenopus*. Our results confirm that cyclophosphamide is not an inducer of cytochrome P-450 in *Xenopus* larvae liver. Compared to the basal activity of the larvae reared in the negative DMSO control, B(a)P induced a clear dose-related response with both endpoints. For instance, a concentration of 0.1 mg/l B(a)P in water induced an EROD activity in the larval liver of approximately 50 pmoles/min/mg protein and produced a significant increase of erythrocyte micronuclei.

4. Discussion

The measurement of chemical parameters of the River Dadou revealed a regular increase of manganese and fluorine between the upstream and the downstream sites. For instance, at point A, manganese and fluorine concentrations were usually below 0.05 and 1 mg/l, respectively, but were as high as 1.46 mg/l for manganese and 2.49 mg/l for fluorine at point H, downstream of the dam. Electrical fishing performed during water sampling highlighted the evolution of fish populations from point A to point H in the river. At the upstream point, the fish inventory corresponded to that of a normal fish population in an uncontaminated river (minnows 42%, gudgeons 31%, trout 21%, lampreys 6%). No fish were found at point D, downstream of the mining activity outlets. Downstream of the dam, at point H, only minnows were captured (estimated population: 2429/ha). These data emphasize the abiotic character of the River Dadou around the Rassisse dam and the low biodiversity in fish populations observed downstream of this point.

The measurement of physical parameters of water samples before testing with amphibians demonstrated the highly acidic character (pH 3.95) of water from point C and the high pH (9.92) of water samples from B. These samples and the industrial samples (F and G) produced markedly toxic effects in the *Xenopus* larvae exposed to it, so that it was not possible to perform the bioassays directly on pure effluents. These observations suggest the probable implication of the industrial activities in the local contamination of the river water.

One direct consequence of the toxic effects observed in amphibian larvae reared in the industrial effluents was the difficulty of revealing the genotoxic potential of water samples using the micronucleus assay. As a result of the toxic conditions, mitotic indices scored in RBCs of the larvae were lower than those of larvae raised in river water and in control groups. Larvae reared in industrial effluents from the mining activity (B, C, F) exhibited a doubling of median values of the micronucleated erythrocyte levels compared to the negative control, even though the differences were

Table 3
Enzymatic activities in the liver and micronucleus formation in erythrocytes of *Xenopus* larvae subjected to increasing concentrations of B(*a*)P

	Negative control	DMSO control	CP 20 ppm	BaP 4 ppb	BaP 40 ppb	BaP 100 ppb	BaP 200 ppb
Liver enzymatic activity							
EROD activity (pm/min/mg protein)	18.1 ± 10.8	11.1 ± 6.3	$10.1 \pm 2.6^*$	$18.3 \pm 1.2^*$	$23.8 \pm 3.4^{**}$	$50.4 \pm 3.9^{**}$	$61.8 \pm 33.9^{***}$
EROD induction	1.63	1	0.91	1.65	2.14	4.54	5.57
Micronucleus assay							
Median	1	1	8	1	3	5	11
Confidence limit	(0.19–1.81)	(0.3–1.7)	(5.57–10.43)	(0.83–1.17)	(2.3–3.7)	(3.74–6.26)	(8.24–13.76)
Result			+	−	+	+	+

EROD activities are presented as mean values with their standard deviations calculated from three assays. Statistical analysis of the results was performed using the Student test. DMSO control was used as reference. Micronucleus assay results are presented as the median values of micronucleated erythrocyte frequencies (per 1000 cells) in circulating blood of the larvae and their 95% confidence limits. DMSO control was used as reference for B(*a*)P genotoxicity evaluation.

* Not statistically different from the control group.

** Statistically different from the control group ($P < 0.05$).

*** Statistically different from the control group ($P < 0.01$).

not statistically significant. The genotoxic potential was probably masked by effluent toxicity, via the reduction of cell divisions in anaemic animals. Given the dilution required for the tests, the real genotoxic impacts of raw effluents were clearly underestimated.

Larvae reared in water sampled at the outlet of the filter-washing process of the plant (G) gave clearly positive responses in the amphibian micronucleus assay. This effluent was enriched in flocculated sludge but, since it was found not to be as toxic as the effluents from the mine, was tested directly. The positive responses obtained were corroborated by the detoxifying activities measured in the liver of the larvae, suggesting the presence of inducing substances (PAHs, PCBs,…) in the water or in the sludge. These potential contaminants are generally poorly soluble substances, mainly adsorbed on particulate matter in the raw water. They are thereby rendered less available for the larvae, which is no doubt a partial explanation for the low toxicity observed in the animals. This low toxicity, in turn, could probably partly account for the significant induction of micronucleated erythrocytes observed in the larvae reared in the filter-washing water of the plant.

Most of the water samples taken along the River Dadou and at outlets from industrial sources induced detoxifying activity in the liver of the larvae, except for samples B and D. In both cases, the negative results observed could be attributed (B) to the dilution of the effluent before testing and (D) to the dilution of the effluent in the river. The highest induction level was measured in larvae reared in the effluent of the mine purification plant (F), after dilution (250 ml/l). Considering the dilution level of the tested effluent and its high toxicity towards *Xenopus* larvae, it can be considered as one of the most biologically active samples in our experiments and may contribute to explaining the very low diversity in fish populations observed downstream of the outlet of the water purification plant of the mine. The high level of EROD induction measured suggests the presence of inducing pollutants in the water samples tested. The presence of PAHs in effluent F could be attributed to products of the extraction process at the mine (fuel from the extracting machines, oil,

waste from the boring wells). The purification plant also treats washing waters from the mining site and from the surrounding soils, which are liable to be contaminated with PCBs. Similarly, high EROD activities were measured in amphibian larvae reared in water sampled in the Rassisse reservoir, receiving water from the River Dadou and from the upstream outlets of the mine (B, C). In this case, the measured induction could probably be attributed to the pollutant concentration in the water and sediments of the reservoir. In the rural environment studied, the principal sources of water contamination likely to induce EROD activities are pesticides and other chemicals applied to the surrounding fields. Other potential sources of EROD-inducing contaminants are forest or field fires, which release PAHs into the air. Indeed, dry and wet atmospheric fallout may also lead to the transfer of pyrolytic hydrocarbon contaminants to the water, either directly or indirectly via soil leaching or run-off in the river.

With the aim of estimating the possible equivalent concentration of these organic contaminants in the river water, experiments to compare EROD activities in *Xenopus* larvae exposed to various B(a)P concentrations were carried out. We showed that river water samples (A, D, H) led to enzymatic activities equivalent to those measured in larvae exposed to a B(a)P concentration of 0.1 mg/l. Likewise, the highly toxic effluent F led to enzymatic activities equivalent to those found in larvae exposed to B(a)P concentrations above 0.2 mg/l. These results have to be considered very circumspectly. Indeed, the hypothetical presence of EROD activities inducers in the water cannot be directly correlated with the detoxifying activities observed in animals exposed to a single substance such as B(a)P. In river water, potential contaminants interact with natural and anthropogenic substances as a whole. In such complex mixtures, synergistic and/or antagonistic interactions between substances lead to biological effects that are not easily predictable. For example, Van Der Oost et al. (1994) could not directly correlate the level of EROD activity in fishes with the concentration of potential inducers of these activities (PCBs, HAPs, organochlorinated substances) in the river water studied. Monod (1997) demonstrat-

ed the inhibition of monooxygenase activities in the presence of high concentrations of hepatotoxic inductors and organic or metallic inhibitors. Biagianti-Risbourg (1997) suggested that some apparent enzymatic inhibitions measured in the livers of exposed organisms may be correlated with the toxicity of the tissue sampled. In the same way, we observed a correlation between relatively low EROD induction levels and small haemolytic livers in larvae exposed to effluent C. Vindimian and Garric (1989), Lindström-Seppa and Oikari (1990) and Adams et al. (1992) demonstrated that complex interactions between substances in water may lead to higher EROD activities in fish sampled downstream of the outlet points, compared to those measured in fish directly sampled at the pollution site. We observed the same type of result using the amphibian model in the water reservoir and at downstream point H. Other authors have concluded that the presence of inhibitory substances in the industrial effluent could explain the differences in induction levels observed in fishes exposed to a pulp mill effluent (Huuskonen and Lindström-Seppa, 1995). Similarly, we postulate the presence of antagonistic effects in river water to explain the results observed with the micronucleus assay and the EROD induction test, both in the River Dadou and in laboratory experiments with B(a)P. Indeed, at the highest concentrations tested, B(a)P induced both a high level of EROD activity and a high level of micronucleated erythrocytes in the larvae. However, for an equivalent EROD induction level measured in the larvae reared in river water samples, the micronucleus assay generally gave negative responses. This apparent contradiction can be explained by the simultaneous presence in the river water of substances that produce antagonistic biological effects. Thus, the presence of high concentrations of contaminants (manganese and fluorine) at the downstream point H, associated with high induction of EROD activities in *Xenopus* livers (probable concomitant presence of organic inductors in river water), could contribute to the observed inhibition of genotoxic effects in the exposed animals. Furthermore, the simultaneous presence of these contaminants in river water could explain the toxic effects observed in *Xenopus* larvae, notably, reduced cell division in circulating

blood, leading to negative responses in the micronucleus assay. The same physiological effects may have contributed to the observation of low population levels of fish in the watercourse section studied.

Factors other than the level of chemical contaminants may affect EROD activity in tested organisms. Indeed, in in situ studies, some authors have demonstrated the modulatory influence of biotic (sex, species) and abiotic (temperature, season) factors on the levels of cytochrome P4501A and/ or associated enzymatic activities (Andersson and Förlin, 1992; Monod, 1997). In our study, the standardized protocol used involved selected and homogeneous groups of amphibian larvae, so that some of the factors of potential variability were eliminated. For instance, Ankley and Blazer (1988) demonstrated in laboratory experiments the influence of food on the monooxygenase activities and their levels of induction in fish. In our study, all amphibian larvae, including the controls, were fed with standardized dehydrated fish food. Likewise, water temperature was constant during exposure of the animals (22 ± 0.5 °C), so that the influence of the variations of this parameter (Andersson and Förlin, 1992) on EROD activities was abolished. Directly related to temperature variations, seasonal changes have been shown to influence EROD activities in numerous in situ studies using different fish species (Jimenez and Stegemen, 1990; Vindimian et al., 1991; Andersson and Förlin, 1992). The sex and age of the animals may also influence the induction of the enzyme (Masfaraud et al., 1990). Some studies on monooxygenase activities in the fish *Cyprinus carpio* demonstrated that juveniles were more sensitive than mature adults (Singh et al., 1985). Consequently, some authors recommend the use of juveniles to measure detoxifying activities, as a means of eliminating variations of activities linked to the sex of the exposed animals (Collier et al., 1995). In our study, young *Xenopus* larvae (15 days old, before metamorphosis) were used at stage 50 of the developmental table of Nieuwkoop and Faber (1956). Other authors have suggested the use of caging experiments with fishes to study river waters (Haasch et al., 1993). This test procedure enables biomarkers to be used in biologi-

cally homogeneous groups of animals, thereby limiting the source of variability due to movement, and allows some of the parameters previously described to be better controlled. In the present study, amphibian larvae were exposed to water samples according to a standardized procedure. Under these conditions, most of the parameters potentially capable of introducing variability in the response levels were under control. Potential variations introduced by the sex of the animals (uncontrolled parameter) in the protocol performed is probably limited by the use of young larval stages, where enzymatic induction levels are less sex-dependent than that in mature organisms (Singh et al., 1985).

5. Conclusion

At the different sampling sites along the river studied here, we observed global toxic effects on *Xenopus* larvae exposed to water samples of industrial origin. Fish inventories performed at the same points demonstrated a general decrease in the population diversity downstream of the mining activity, correlated with the presence of high concentrations of manganese and fluorine in the water. In previous studies, these two substances were not found to be genotoxic using in vivo (Fernandez et al., 1993) and in vitro test systems (Li et al., 1988; Ashby and Tennant, 1991). Using *Xenopus* larvae, we did not find any particular genotoxic effects, except in animals exposed to water from the filter-washing process of the water treatment plant. In most cases, the high toxicity of industrial effluents masked the potential genotoxic effects, since diluted effluents were needed to perform the tests. Concerning the effluent from the water treatment plant, the high concentration of flocculated sludge at the outlet point of the effluent in the river, associated with the observed genotoxic effects, suggests the presence of genotoxic contaminants adsorbed on the particulate matter of the waste.

A supplementary level of information was provided by the EROD activity measured in the livers of *Xenopus* larvae exposed to the tested waters. Thus, most of the water samples induced high levels of detoxifying activities in the experimental larvae, compared to those reared in water sampled

upstream of industrial activities in the River Dadou. This observation suggests that potentially inducing substances were present in the river water. Classical inductors of the cyt P450IA1 detoxifying systems, commonly found in the environment, include the polyaromatic hydrocarbons, polychloro biphenyls and dioxins. Such substances may be produced by the local industrial activities or imported from the surrounding areas by atmospheric pollution or by the river via contaminated soils (PCBs, pesticides, chlordane).

To date, due to the characteristics of the industrial activity, only potentially metallic contaminants (Al, Cu, F, Mn) have been measured in the water of the River Dadou. The results obtained in this study suggests that it is important to monitor supplementary parameters, such as PAHs and PCBs, considering, respectively, the potential release from the mining activity and the general rural situation of the studied region.

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