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Amphibian Micronucleus Test *in Vivo* (Jaylet Test) to Evaluate the Genotoxicity of Petrochemical Waste Waters

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For several years, chemical analysis have been commonly employed in aquatic environmental studies. These techniques cannot actually detect and quantify all pollutants present in water samples. Furthermore, chemical methods cannot take into account many environmental phenomena such as bioavailability, bioaccumulation, synergistic and antagonistic effects between the compound species. For these reasons, many investigations for evaluating the effects of xenobiotic on organisms use *in vivo* and/or *in vitro* bioassays. However, for *in vitro* tests, samples often need to be concentrated and their genotoxic effects are estimated on the extracts. Such pretreatments can modify the chemical nature of the pollutants contained in the complex mixtures (for example the waste waters), without the exact repercussion on the subsequent genotoxic effects being known. Thus, one of the best ways to estimate the risk assessment of pollutants in environment is to use biological tests *in vivo*, which give a global response of all chemicals present in the medium. In genetic ecotoxicology nowadays, *in vivo* genotoxicity tests for aqueous and sediment or soil samples are widely used with various organisms such as plants (De Marco and De Simone 1994), molluscs (Scarpato et al. 1990), and vertebrates (Gauthier et al. 1990). The Jaylet test was used for this research, since by virtue of its sensitivity, it constitutes an interesting tool for detecting most mutagens found in complex mixtures (review: Ferrier et al. 1998).

Djomo et al. (1995) reported for amphibian larvae (*Pleurodeles waltl*) the genotoxicity of some major polycyclic aromatic hydrocarbons (PAHs) found in crude oil, refined on a petrochemical site situated in Lacq town (Southwest France). The present investigation attempts to evaluate the genotoxic effects of waste waters of the same site which proceed from industrial processes after physicochemical and biological treatments.

MATERIALS AND METHODS

After laying, the eggs of *Pleurodeles waltl* are placed at 20 ± 0.25 °C in dishes containing 5-6 liters of tap water previously filtered over sand and activated

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charcoal. The temperature control during rearing is important since growth is temperature-dependent, and 20 °C is an optimal temperature for development. The water used to rear larvae was prepared in the laboratory from water that was dialysed and deionized in a Milli Q system (Millipore Corporation, USA). It was then enriched with essential minerals that are normally found in tap water to the following concentrations per liter: 294 mg $CaCl_2$, $2H_2O$; 123.25 mg $MgSO_4$, $7H_2O$; 64.75 mg $NaHCO_3$ and 5.75 mg KCl. Two months after laying, larvae are ≈ 30 mm long, and they reach 40 mm within the next 12 days. This is a suitable time for taking blood samples by cardiac puncture since it represents a period of rapid growth and the proportion of mitoses in peripheral red blood cells is ≈ 10%. The micronucleus test was performed on amphibian larvae (*Pleurodeles waltl).* It is based on a comparison (after 12 days of treatment) between the levels of micronucleated erythrocytes in blood smears of larvae reared in water containing a clastogenic substance and control larvae.

The larvae used for the assay were taken at the stage when the fifth toe on the hind foot is just outlined. For each experiment, several groups of 15 larvae were constituted from the same hatching. Animals were reared at 20 ± 0.25 °C in glass flasks containing: (i) 1.5 L of 'reconstituted' water used for negative and positive controls (100 mL per larva); (ii) the effluent to be tested (absent in the controls); (iii) food (*Daphnia*). For positive control, cyclophosphamide monohydrate was used (lot n° 73H0846, Sigma, St louis, MO, USA). Larvae were alternately exposed to subdued natural light and to darkness (day/night rhythm).
Preliminary toxicity experiments were carried out with the aim of determining the maximum concentration (MC) of the effluent to be used in the Jaylet test. MC corresponded to the half maximum dose which did not induce detectable physiological disturbances after 6 days of treatment.

The genotoxicity test was carried out under the same conditions as described above. At the end of the treatment period (12 days), a blood smear was taken from each animal. It was fixed in methanol (3 min), stained with Masson's hemalum (10-12 min), rinsed in running water (10 min), and dried. The slides were examined under a microscope equiped with an oil immersion lens (× 1000).

For each animal (one blood smear per animal), four lots of 250 cells were counted. In order to take into account a possible heterogeneity of the smear, the four fields were chosen in topographically separated areas of the slide. Thus, a total of 1000 cells were counted per larva, which is the minimum required for satisfactory statistical appreciation of the results. The levels of micronucleated erythrocytes from 'n' animals in a group were ranked in ascending order. The characteristic values of the samples (the median, the lower and the upper quartiles) were calculated. Statistical analysis was carried out according to McGill et al (1978). If *M, IQR* and *n* correspond respectively to the median, interquartile range and sample size ($n \geq 7$), the 95% confidence interval of median is expressed by :

$$M \pm 1.57 \times IQR / \sqrt{n}$$

The difference between two medians *M1* and *M2* is significant at 5% level if the two calculated confidence intervals do not overlap (the result is considered positive). The industry whose waste waters were tested, refines crude oils and also produce sulphur and chlorine. The effluent tested was essentially composed of waste waters from industrial processes (run-off, cooling and washing waters). The samples were collected in September 1995 at three sites: the waste waters from discharge point after physicochemical and biological treatments (decantation, neutralization), the water without petrochemical effluent taken from the Midouze River, southwest of France (≈ 2000 m upstream of effluent outlet) and effluent mixed to Midouze water (≈ 500 m downstream of effluent outlet). The samples were stored in tanks, transported to the laboratory within 24 hours and cooled to 4 °C until use. Chemical analysis were carried out by HPLC or GC/MS (for the aromatic compounds) and by Spectrometer (for the mineral compounds) on samples previously filtered at 0.45 µm (filter type Whatman GF/G).

RESULTS AND DISCUSSION

The chemical analysis (Table 1) showed that some compounds such as nitrite (NO_2^-), sulphate (SO_4^{2-}), chlorine (Cl^-) and total PAHs are present at high levels in the samples. Their concentrations in water collected at the discharge point are about 2-fold (NO_2^-), 1.5-fold (SO_4^{2-}), 2-fold (Cl^-) and 3.2-fold (total PAHs) higher than those found in downstream. In preliminary bioassays, larvae were exposed for 6 days to increasing concentrations of each test waste water. At all concentrations tested, no toxic effect was observed with water collected upstream or downstream. Larvae reared in presence of the effluent taken at discharge point (at 1000 mL/L) showed a light inability to catch and eat *Daphnia*. The results concerning the genotoxicity test (Table 2) revealed that for the effluent from discharge point, positive responses were obtained at 250 and 500 mL/L with a dose effect. Thus, compared to the negative control, the levels of micronuclei (MN) observed at 125, 250, and 500 mL/L were, respectively, 1.5-fold, 3-fold and 6.5-fold higher. The formation of MN may be attributed to the action of many classes of aromatic and mineral compounds whose concentrations have been illustrated in Table 1. Houk (1992) assumed that in industrial waste waters some mineral compounds such as nitrite, chlorine, phosphate and sulphur could combine with aromatic compounds to form nitrated, chlorinated, sulphonated and/or phosphorated aromatic compounds whose genotoxicity have been demonstrated on various organisms. Among these complexed aromatic compounds, the 1-nitropyrene, dinitropyrenes and methylphenanthrenes were used in several assays. For example, the exposition of *Salmonella typhimurium* to three Canadian petrochemical effluents revealed that more than 58% of mutagenic activities were due to 1-nitropyrene and to methylic aromatic compounds (methylnaphthalene and methylphenanthrene), (Houk 1992). In cytogenetic tests carried *in vivo* on mouse bone marrow cells and *in vitro* on chinese hamster ovary cells (CHO), treated with petrochemical effluents, these compounds were also identified as the main chemicals responsible for the chromosomal damage and pertubation of eukaryote

cell division (Latt et al. 1977). The sister chromatid exchanges on CHO cells and structural aberrations were attributed to these chemicals by De Marini et al. (1987) during the test of clastogenicity.

The genotoxicity of the effluents may be also due to some mineral compounds, present at high levels, such as free chlorine up to 0.5 g/L as shown by chemical analysis (Table 1). Indeed, Gauthier et al. (1990) found positive responses on *Pleurodeles waltl* exposed to water treated by chlorination procedures. Recently, Nylund et al. (1994) used the Ames test and the SOS chromotest to evaluate the genotoxicity of kraft pulp spent liquors from different types of chlorination procedures. In both tests, these authors observed that the decrease in genotoxicity corresponded to the decrease in chlorine levels.

Table 1. Chemical analysis of waters collected at the discharge point and downstream.

Parameters		Discharge point	Downstream
Conductivity	($\mu S/cm^2$)	3.80	3.02
pH		8.10	7.90
Suspended matters		25.10	23.50
Alcalinity		92	56.30
PO_4^{3-}		14	8.0
NO_2^-		40.80	19.70
NO_3^-	(mg/L)	8.20	5.40
SO_4^{2-}		1382.0	970.50
Cl^-		506.0	260.0
F^-		10.0	8.20
Phenanthrene		28.40	11.0
Anthracene		7.0	1.0
3-Methylphenanthrene		4.20	0.50
2-methylphenanthrene		5.40	1.60
9-Methylphenanthrene		4.0	1.20
1-Methylphenanthrene		3.40	0.30
Fluoranthene	(ng/L)	3.30	1.30
Pyrene		3.80	1.20
Chrysene		1.40	0.70
Benzo(a)pyrene		0.80	0.50
benzo(e)pyrene		0.60	0.50
Benzo(f)fluoranthene		1.90	0.0
Total PAHs		64.20	19.80

Table 2. Frequencies of micronucleated red blood (per 1000 cells) in larvae treated with different concentrations of a petrochemical effluent.

	Concentration (mL/L)	Micronuclei frequency					Mean	no of animals	Result[b]
		Lower extreme	Lower quartile	Median ± confidence limit	Upper quartile	Upper extreme			
Negative control	0	2.0	5.0	8.0 ± 1.62	9.0	14.0	7.60	15	-
Positive control[a]	2	7.0	13.0	16.0 ± 3.24	21.0	32.0	18.46	15	+
Upstream	1000	1.0	5.0	9.0 ±1.62	9.0	12.0	6.80	15	-
Discharge point	125	7.0	9.0	11.0 ± 1.67	13.0	17.0	11.07	15	-
	250	16.0	18.0	24.0 ± 5.03	30.0	44.0	26.14	15	+
	500	40.0	41.0	52.0 ± 7.94	57.0	72.0	51.60	15	+
Downstream	500	1.0	6.0	8.0 ± 0.83	8.0	10.0	6.28	15	-
	1000	1.0	6.5	8.0 ± 0.60	8.0	12.0	6.93	15	-

[a]Cyclophosphamide monohydrate in (mg/L). [b] + : Positive result, - : negative result

In addition, our bioassay was carried out on unfiltered samples, whereas the chemical analysis were performed on water previously filtered at 0.45 µm. Therefore, the chemical data did not reveal the presence of all genotoxins present in the test effluent, since some of potency pollutants could have been adsorbed on suspended materials (Narbonne et al. 1999), and was probably eliminated by filtration. For example, Djomo (1996) found that filtration entirely eliminates some PAHs with high molecular weight such as dibenzothiophenes and benzo(a)anthracene (BaA). The genotoxic effects of BaA from 1.10 to 3.28 ppb and its derivates (7, 12-dimethylbenz(a)anthracene from 0.05 to 0.19 ppb) was observed on the *Pleurodeles waltl* by Fernandez et al. (1993); positive responses were also obtained *in vivo* on mouse and *in vitro* on *Salmonella typhimurium* (strain TA 100) in presence of metabolic activation of S9 by Ito et al. (1988). Likewise, this compound induced genic mutation on *Drosophila melanogaster* (Frölich and Würgler 1990).

With waters collected upstream or downstream, negative responses (no significant increase in MN) were found at all concentrations tested. Thus, the negative results obtained on water taken upstream of the discharge point showed that the upstream constitutes an interesting point of reference. In the case of water taken downstream, the results could be due to the fact that the genotoxic substances contained in the discharge have been diluted too much by the water of Midouze River to bring about a positive result in the Jaylet test. It is also suggested that antagonistic relationships exist between certain substances contained in the waste water and substances present in the water of Midouze River. Indeed, in aquatic environment most pollutants become rapidly associated with particles such as humic matter, and suspended solids and they are deposited in sediment (Djomo et al 1996; Narbonne et al. 1999). The results obtained in this study suggest the sensitivity and the ecotoxicologic relevance of the amphibian micronucleus test *in vivo* (Jaylet test). Thus, its future use by the organizations responsible for supervising the discharge of waste into watercourses should be extended.

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REFERENCES

De Marco A, De Simone C (1994) The influence of humic substances in seeding soil on the genotoxic effects induced in *Vicia faba* by the herbicide Maleic hydrazide. Fresenius Environ Bull 3:6-11.

De Marini DM, Brusick DJ, Lewtas J (1987) Use of limited protocols to evaluate the genotoxicity of hazardous wastes in mammalian cell assays: comparison to *Salmonella*. J Toxicol Environ Health 22:225-239.

Djomo JE (1996) Ecotoxicité en eau douce de certains hydrocarbures aromatiques

polycycliques (HAPs) majeurs d'un pétrole brut: effets sur quelques organismes aquatiques tests. Thèse de Doct. Univ. Paul Sabatier, Toulouse (France).

Djomo JE, Garrigues P, Narbonne JF (1996) Uptake and depuration of polycyclic aromatic hydrocarbons from sediment by the zebrafish (*Brachydanio rerio*). Environ Toxicol Chem 45:1177-1181.

Djomo JE, Ferrier V, Gauthier L, Zoll-Moreux C, Marty J (1995) Amphibian micronucleus test *in vivo*: evaluation of genotoxicity of some major polycyclic aromatic hydrocarbons found in crude oil. Mutagenesis 10:223-226.

Ferrier V, Gauthier L, Zoll-Moreux C, L'Haridon J (1998) Genotoxicity tests in amphibians – A review. In: Wells et al. (ed), Microscale Testing in Aquatic Toxicology : Advances, Techniques and Practice. CRC Press LLC. Boca Raton, London, New York, Washington, D.C., pp. 507-519.

Fernandez M, L'Haridon J, Gauthier L, Zoll-Moreux C (1993) Amphibian micronucleus test(s): a simple and reliable method for evaluating *in vivo* genotoxic effects of freshwater pollutants and radiations. Initial assessment. Mutat Res 292:83-99.

Frölich A, Würgler FE (1990) *Drosophila* wing-spot test: improved detectability of genotoxicity of polycyclic aromatic hydrocarbons. Mutat Res 234: 71-80.

Gauthier L, Lévi Y, Jaylet A (1990) Application du test micronoyau triton à l'étude directe de la génotoxicité des procédés de désinfection des eaux. J French Hydrol 21:147-157.

Houk VS (1992) The genotoxicity of industrial wastes and effluents. Mutat Res 277:91-138.

Ito YN, Ueda S, Maeda S, Murao T, Sugiyama H, Harvey RG (1988) Induction of chromosomal aberrations in rat bone marrow cells and mutations in *Salmonella typhimurium* by benzo(a)anthracene derivatives. Mutat Res 206:55-63.

Latt SA, Allen JW, Rogers EE, Juergens LA (1977) *In vitro* and *in vivo* analysis of sisters chromatid exchange formation. In: Kilbey et al. (ed), Handbook of Mutagenicity Test Procedures. Elsevier, Amsterdam, pp. 275-291.

Mc Gill R, Tuckey JW, Larsen WA (1978) Variations of box plots. American Statist 32:12-16.

Narbonne JF, Djomo JE, Ribera D, Ferrier V, Garrigues P (1999) Accumulation Kinetics of polycyclic aromatic hydrocarbons adsorded to sediment by the mollusk *Corbicula fluminea*. Ecotoxicol Envion Saf 42:1-8

Nylund L, Rosenberg C, Jappinen P, Vainio H (1994) Genotoxicity of kraf pulp spent liquors from different types of chlorination procedures. Mutat Res 320:165-174.

Sparcato RL, Migliore G, Alfinito C, Barale R (1990) Induction of micronuclei in gill tissue of *Mytilus galloprovincialis* exposed to polluted marine waters. Mar Poll Bull 21:74-80.