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Genotoxicity of River Water under the Influence of Petrochemical Industrial Complexes

C. T. Lemos,[1] V. M. F. Vargas,[1] J. A. P. Henriques,[2] M. S. Mattevi[3]

[1]Fundação Estadual de Proteção Ambiental, Av. A. J. Renner, 10, 90250 Porto Alegre, RS, Brazil
[2]Department of Biophysics and Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[3]Department of Genetics, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

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The toxic effects of industrial wastes discharged into natural waters should be intensively investigated since they may affect the survival, behavior or genetic composition of aquatic organisms, as well as the health of the population drinking this water.

Most mutagenic, carcinogenic and mutagenic-carcinogenic substances can be detected by tests which evaluate alterations in DNA sequence in combination with at least one in vitro test (Brusick, 1988; Stahl, 1991). Among the methods used to determine the genotoxicity of a substance, the Ames test on bacteria and analysis of sister chromatid exchanges (SCEs) in lymphocytes are considered to be classic. The Ames test has been extensively used to determine the mutagenicity of environmental samples, among them river water (Vargas et al, 1988; Valent, 1990) and industrial effluents (McGeorge et al, 1983; Metcalfe et al, 1985; Vargas et al 1988). SCE analysis has shown considerable potential for the detection of mutagens and carcinogens in human populations exposed to different genotoxic conditions, including polluted natural waters (Alink et al, 1980).

The present report presents the results obtained using these two methods for the evaluation of the genotoxicity of water from the Caí River, in the area affected by the Petrochemical Complex of the State of Rio Grande do Sul, Brazil. After treatment, the wastes of the complex are discharged into the Caí River, an important tributary of the Guaiba River, which provides the drinking water used by the approximately 1,200,000 inhabitants of Porto Alegre, capital city of the State of Rio Grande do Sul.

MATERIALS AND METHODS

The rainwater draining channels and the industrial effluents of the III Petrochemical Complex are discharged directly or indirectly into the Caí River (figure 1). Water samples were collected from the river at two sites, Ca.18.6 and Ca.13.6). Ca.18.6 is close to the area of final disposal of fluid industrial waste and Ca.13.6 is located in the areas of disposal of the accumulation and safety basins, receiving also rainwater drainage. Five samples were collected at Ca.18.6 and six at Ca.13.6 according to the Standard Methods for the Examination of Water and Wastewater (1985) over a period of 10 months.

Correspondence to: C. T. Lemos

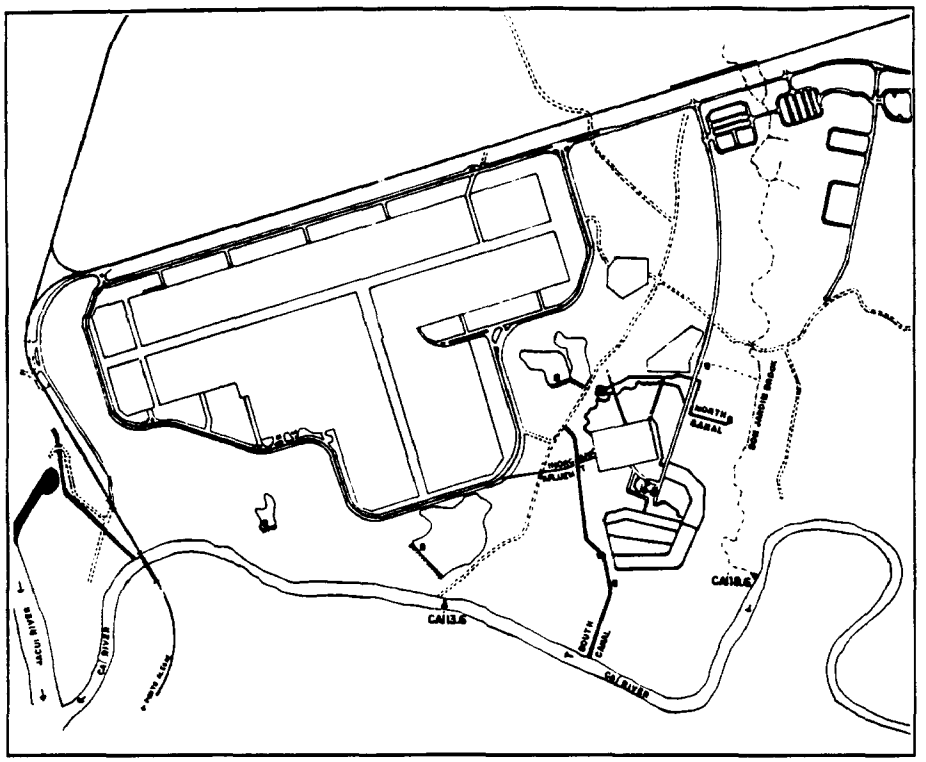

Figure 1. Sampling sites on the Cai river affected by the Petrochemical Industrial Complex of Rio Grande do Sul.

Lymphocytes from a single donor, a healthy non-smoking individual with no history of serious disease, were used in parallel cultures (RPMI-1640 medium, 10 μg/ml BrdU) for SCE determination of test and control samples. After 48 hr of incubation, the cultures received 200 μl of the water to be tested and paired controls received 200 μl distilled water. Two hr later the media were changed and cultures were again incubated for up to a total of 72 hr. Slides were stained with Hoescht 33258 by the method of Perry and Wolf (1974). Twenty-five metaphases with asymmetrical chromosomes per test sample and respective control were analyzed by a blind test and the mean numbers of exchanges per cells were compared by the Student t test. The lymphocyte cytotoxicity was measured by the mitotic indexes of the test and control samples.

The organisms used in the Ames test were strains TA98 and TA100 of *Salmonella typhimurium* (kindly provided by Dr. B. Ames, Berkeley, CA) which detect mutagens that alter DNA reading (frame shift) and cause base pair substitutions, respectively.

The mutagenicity test was carried out by the preincubation procedure (see Maron and Ames, 1983) modified according to the protocol of the Institute of Medical Research (1983) and previously described by Vargas et al (1988). Cytotoxic analysis and estimates of true revertant colonies (His+) were performed according to Maron and Ames (1983) and

Vargas et al (1988). The result was considered to be positive when the number of revertants per sample plate was double the number detected in the negative control (Ames et al, 1975; Maron and Ames, 1983). The positive results were confirmed by repetition and reproducible dose-response curve (Vargas et al., *in press).* The following positive controls were used in each test: in the absence of S9 mix, sodium azide (5 μg/plate) and N-oxide 4 nitroquinolein (4 NQO, 0.5 μg/plate) for the TA100 and TA98 strains, respectively; in the presence of S9 mix, aflatoxin B1 (0.5 μg/plate) for both strains. The negative control used was 200 μl sterile distilled water. Water samples (2,000 or 1,000 μl) from the Caí River were added to 100 μl bacterial test-cultures in the presence or absence of the metabolizing fraction (S9 mix) and inoculated using surface agar at twice the concentration (Vargas et al, 1988). The S9 fraction was prepared from liver homogenates of Sprague-Dawley rats pretreated with Aroclor 1254 according to the protocol of Ames et al (1975).

RESULTS AND DISCUSSION

Table 1 shows the results of the Ames test obtained with strain TA98 of *S. typhimurium* in the presence and absence of a metabolizing fraction (S9 mix). In the direct assay, positive activity was observed in 4 samples. After metabolization, mutagenic activity persisted in only one sample (Ca. 18.6 II). Samples Ca. 13.6 II and V showed positive activity only in the assay with metabolic activation. When samples with mutagenesis indices of 1.4 and 1.8 were tested by the Student t test, significant responses ($p<0.05$) were detected in Ca. 13.6 V in the direct assay and in Ca. 18.6 IV and Ca. 13.6 IV after metabolization ($p<0.05$), a fact which was considered to indication of mutagenicity . Cytotoxic effects were also observed in Ca. 13.6 II in the presence of S9 mix in the assay with 2,000 μl, but not in the assay with 1,000 μl.

Table 1. Analysis of the mutagenicity of 11 water samples from the Caí river using the TA98 strain of *S. typhimurium*.

Samples		TA98				TA98 S			
		M	I	R/pl	S%	M	I	R/pl	S%
13.6	I	+	2.1	65±5	100	-	0.9	40±3	92
13.6	II	-	0.9	37±9	100	-	1.0	34±1	30
13.6	II*			NT		+	3.8	137±2	100
13.6	III	-	0.7	28±2	100	-	0.5	19±1	100
13.6	IV	-	0.8	31±3	80	-	1.4	50±3	100
13.6	V	-	1.8	72±8	100	+	2.2	86±10	93
13.6	VI	+	4.0	161±4	100	-	1.4	55±4	86
18.6	I	-	0.7	20±7	100	-	0.5	23±1	90
18.6	II	+	3.0	119±4	80	+	2.6	93±16	100
18.6	III	-	0.9	35±3	100	-	1.0	36±7	100
18.6	IV	-	1.2	44±5	100	-	1.4	52±3	73
18.6	VI	+	6.7	266±44	87	-	1.8	77±10	91

M: mutagenicity; I: mutagenesis index (number of revertants obtained in the sample/ number of revertants of the negative control); R/pl: revertants per plate; S%: percent survival; NT: not tested; *dose/plate 1,000 μl, and 2,000 μl for all others.

Mutagenic activity of the base pair substitution type (TA100) was also examined (data not shown). Ca.13.6 I and V gave positive responses in the direct assay. Ca.18.6 IV and Ca.13.6 IV (respective indices of 1.8 and 1.4), however, showed significant mutagenicity when analyzed by the t test ($p<0.01$).

Table 2 shows the mean number of SCEs per cell observed in human lymphocyte cultures exposed to 11 samples of raw water and in their respective distilled water controls. It is interesting to emphasize the small number of SCEs detected in the controls and in the tests, which consisted of cultured lymphocytes obtained from the same individual for all assays, with a characteristically low baseline ($\pm$4.15). This value agrees with population studies cited by the working group of the Gene-Tox Program/WHO (1985), whose means ranged from 7 to 10 SCEs/cell, with an individual variation of 2 to 45 SCEs/cell in normal populations. The mitotic indexes of three test cultures were lower than their negative controls (Ca.13 III and IV; Ca.18 III). These samples induced different SCE numbers (see Table 2).

Table 2. Mean SCE numbers observed in human lymphocytes treated with 11 water samples from the Caí River and in control distilled water cultures.

Site	Sample	Mean SCE number		Significance
		Test	Control	
13.6	I	5.6±2.5	2.6±2.0	***
13.6	II	8.4±2.3	3.2±1.6	***
13.6	III	4.6±2.2	3.2±1.6	*
13.6	IV	5.0±2.4	2.6±2.0	***
13.6	V	7.8±1.9	4.9±1.8	***
13.6	VI	6.3±2.0	4.9±1.8	*
18.6	I	5.6±1.4	5.9±2.0	NS
18.6	II	8.5±2.4	4.9±1.8	***
18.6	III	4.3±2.7	3.2±1.6	NS
18.6	IV	4.2±1.8	2.6±2.0	**
18.6	VI	9.2±2.9	4.0±1.5	***

NS: not significant; *, **, ***: significant at the 0.05, 0.01 and 0.001 level, respectively.

Nine of the 11 samples tested presented a significant increase in exchanges when compared to their controls. Only samples I and III from site 18.6 showed the same numbers as the controls, although it should be pointed out that the lack of significance observed for sample I was mainly due to the unusually high level observed in its control.

Table 3 compares the results obtained by the Ames test and by SCE analysis. The results in this table were considered to be negative (-) when they did not differ from the controls, and positive (+) when significant t values were obtained for SCE number or when rates at least double those obtained by spontaneous mutation were observed in the Ames test. In the Ames test, the results were considered to be positive (+) when they were observed both

Table 3. Comparison of the results obtained by the Ames test and by SCE analysis.

Site	Sample	Ames test	SCEs
13.6	I	+	+
13.6	II	+	+
13.6	III	-	+
13.6	IV	+/-	+
13.6	V	+	+
13.6	VI	+	+
18.6	I	-	-
18.6	II	+	+
18.6	III	-	-
18.6	IV	+/-	+
18.6	VI	+	+

in TA100 and TA98 with or without metabolic activation. Results were considered to be "intermediate" (+/-) when the Ames test gave some indication of mutagenicity.

Concordant and positive results by the two tests were obtained at Ca.18.6 II and IV and Ca.13.6 I, II, V and VI, and negative results were obtained at Ca.18.6 I and II. Samples 18.6 IV and Ca.13.6 IV presented significant increases when analyzed by the t test ($p<0.01$, SCEs; $p<0.05$, Ames test), although the mutagenicity indices did not reach a level double the spontaneous mutation rate measured by the Ames test (see table 1). On the other hand, Ca.13.6 III showed increased mean exchange numbers but no mutagenicity by the Ames test.

The samples used in the present study are complex because they come from raw waters which receive industrial wastes and rainwater effluents carrying the washing of the atmosphere close to industrial zones, residues from marginal zones, and the organic load generated by the aquatic ecosystem itself.

Of the 11 samples studied, six gave a positive response to both assays (table 3), indicating the mutagenic activity and the potential for the induction of SCE of these samples. These results can be assigned to the three response categories proposed by Latt et al (1981). Samples I and II from Ca.13.6 and sample IV from Ca.18.6 were strongly positive by these criteria. Moderately positive activity was detected at Ca.18.6 II and Ca.13.6 V which, like the previous sites, had presented positive mutagenicity when tested on bacteria. A similar result was obtained by Metcalfe et al (1985) in samples of petroleum refinery effluents. In contrast, Ca.13.6 VI was positive in the direct assay in the Ames test but induced SCE responses different from the control only at the significance of 0.05. This sample, according to Latt et al (1981), should be classified as temporarily positive for SCE induction.

Positive mutagenic activity in the Ames test is considered to be important since negative results may also be due either to sample toxicity (Loper and Lang, 1978) or to the presence of substances to which the strains employed are not sensitive. Toxicity can be controlled by

analysis of survival curves. In this respect, Ca.13.6 II proved to be highly toxic in the assay performed with 2,000 µl of the sample, an effect that disappeared with the use of 1,000 l, thus demonstrating the mutagenic effect of the sample.

Interestingly, some samples (Ca.13.6 II and V) only showed mutagenicity by the Ames test after metabolization but induced increased SCE numbers without the microsomal fraction. Menhert et al (1984) suggested that lymphocyte cultures can metabolize some premutagens but not others. Similar results have been obtained by Cid and Mattos (1984) in a study of lymphocyte metabolization. It could be also suggested that different substances may be identified within the complex sample studied here as the result of sensitivity to each assay. In contrast, samples Ca.18.6 IV and Ca.13.6 IV which gave moderate and strong positive results, respectively, for SCE induction (by the classification of Latt et al, 1981), were negative in the Ames test. However, when the results of the Ames test were analyzed by the t test, a significant increase was observed when compared to the control. The use of this statistical test for the Ames test was also adopted by Loper (1980), since river samples may present diluted genotoxic substances, causing low mutagenic induction.

Ca.13.6 III, which was negative by the Ames test, was classified as temporarily positive since it did not reach twice the mean SCE number detected in the control despite a significant increase in relation to it. In contrast, the absence of mutagenic activity of samples I and IV at Ca.18.6 permit their negative classification in both assays.

Comparison between the two areas studied, which result from different discharges (18.6 close to the final effluent and 13.6 in the fluvial drainage area, see figure 1), shows production of elevated contamination in both areas by the two tests, but of a worse nature at Ca.13.6 which collects the rainwater drainage of the complex.

Previous evaluation by the Ames test showed that this assay was sensitive to substances which have proved to cause damage to human cells. The lack of a stronger correlation in some cases was probably due to the lower sensitivity of this methodology for the detection of the action of certain substances such as solvents and metals (Rossman et al, 1984). In contrast, the use of SCE production in genotoxic studies shows that the test can efficiently detect alkylating substances which produce DNA adducts (Carrano and Natarajan, 1988) and is extremely sensitive to compounds which cause common chromosome aberrations, a fact indicating their clastogenic potential. Positive results in terms of SCE induction generally indicate that a mutagen is also a carcinogen since the test gives few false-positive results (Latt et al.,, 1981). These associatios are confirmed by the results detected by Ames test (McCann et al, 1975; McCann and Ames, 1976, 1977; Zeiger and Tennant, 1986; Ashby and Tennant, 1991).

These considerations are important because they are of interest in environmental evaluation of natural waters used for public drinking supplies. However, the fact that natural waters present mutagenic-clastogenic activity with or without a relationship with the carcinogenic potential is of itself an important biological factor in the equilibrium of aquatic ecosystems and in the relationship between the different levels

of the food chain.

The present report confirms results obtained by Vargas et al (1988) and reinforces the need for genotoxic monitoring of this water supply by the simultaneous use of different assays, as recommended by Ashby and Tennant (1991).

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