Ecotoxicological assessment model to evaluate industrial effluents using different biological end-points and traditional chemical parameters

Modelo de avaliação ecotoxicológica para avaliar efluentes industriais utilizando diferentes testes biológicos e parâmetros físico-químicos tradicionais

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Abstract: Aim: The purpose of this study was to evaluate acute and chronic toxicity besides the genotoxicity found in samples of the last stabilization lagoon and in the accumulation and safety drainage basins in Petrochemical Complex using organisms with different trophic levels. **Methods:** A sequential, selective ecotoxicological assessment model was applied using different biological end-points and traditional chemical parameters in an area where effluents from a petrochemical industry complex are treated in Rio Grande do Sul, Brazil. Species from different trophic levels were used to evaluate acute, chronic toxicity and genotoxicity in samples from three pluvial drainage basins (B3, B4, B7) and the last stabilization lagoon for liquid effluents (L8) considered sources in this assessment model. **Results:** Acute toxicity tests with *Danio rerio* and *Pimephales promelas* were negative in the first stage, and the traditional chemical parameters showed some sampling values that were different from current Brazilian legal values. Chronic toxicity assays using *Daphnia magna* to quantify mortality, births and ephippia formation were applied based on the absence of positive results for acute toxicity. Increased mortality was observed in the B7 samples, and a high rate of ephippia formation occurred in the B3 and B4 samples, indicating the presence of chronic action contaminants. Since water samples of L8 continued testing negative, we applied the *Salmonella* assay and it confirmed mutagenic events with and without hepatic metabolic activation. In the last phase of the study chronic toxicity effects of compounds were also found deposited in the sediments of this lagoon. **Conclusions:** All results allow the characterization of the release pathway of chemical mixtures that can generate chronic and genotoxic damage in the area of environmental influence.

Keywords: petrochemical complex, *Daphnia magna*, *Salmonella*/microsome, *Danio rerio*, *Pimephales promelas*.

Resumo: Objetivo: O objetivo deste trabalho foi avaliar as toxicidades aguda e crônica além da genotoxicidade em amostras da última lagoa de estabilização de um complexo petroquímico e de bacias de acumulação e segurança deste mesmo Complexo, usando organismos de diferentes níveis tróficos. **Métodos:** Foi aplicado um modelo seqüencial e seletivo utilizando organismos de diferentes níveis biológicos e parâmetros físico-químicos tradicionais em área de um complexo industrial petroquímico situado no Rio Grande do Sul, Brasil. Espécies de níveis tróficos diversos foram utilizadas para avaliar toxicidade aguda, crônica e genotoxicidade em amostras provenientes de três bacias de drenagem pluvial (B3, B4, B7) e da última lagoa de estabilização dos efluentes líquidos do complexo (L8). **Resultados:** No primeiro estágio os testes de toxicidade aguda com Danio rerio e Pimephales promelas, foram negativos e alguns parâmetros físico-químicos tradicionais mostraram valores discordantes do permitido pela legislação brasileira. Ensaios de toxicidade crônica com *Daphnia magna* considerando mortalidade, nascimentos e formação de efípios foram realizados devido à ausência de resultados positivos para toxicidade aguda. Neste nível trófico foi observado aumento na mortalidade nas amostras da bacia B7 e elevada taxa de formação de efípios nas amostras das bacias B3 e B4, indicando presença de contaminantes com ação crônica. Como as amostras da lagoa L8 continuaram negativas foi aplicado o ensaio com *Salmonella* para confirmar eventos mutagênicos com e sem metabolização hepática. Na última fase do estudo foi identificado efeito crônico nos sedimentos de fundo da L8. **Conclusões:** Os resultados permitiram a caracterização da rota de liberação das misturas químicas que podem gerar danos crônicos e genotóxicos na área de influencia ambiental.

Palavras-chave: complexo petroquímico, *Daphnia magna*, *Salmonella*/microsome, *Danio rerio*, *Pimephales promelas*.

1. Introduction

FEPAM-Fundação Estadual de Proteção Ambiental, the environmental protection agency of the southernmost state of Brazil, developed an ecotoxicological assessment model to study the presence of hazardous compounds in industrial effluents. This was applied selectively in sequence, using different biological end-points and traditional chemical parameters (FEPAM/PADCT/FINEP, 1997, 2004; FEPAM, 2008). The evaluation of toxicity by means of the chemical analysis of isolated substances may supply inaccurate data concerning the toxic effect of complex mixtures on aquatic

organisms. Biological tests to classify the environment's capacity to preserve aquatic life and human health have been essential to evaluate the quality of environmental compartments (Terra et al., 2008, Vargas et al., 2008).

Observations of living beings are important because substances that are not detected by chemical analyses may interfere in the organisms, triggering biological responses (Terra et al., 2008). Bioavailability must be evaluated by means of bioassays, since many environmental stressors become visible only when they are present in high doses (Lemos and Terra, 2003). However, when they are in small amounts, they silently affect an individual's genetic baggage, interfere in physiological functions, and modify the reproductive frequency or quality and quantity of generated organisms (Terra and Feiden, 2003). Therefore the purpose of toxicity tests is to express the effects of the interactions of substances found. Biological tests can evaluate the presence of hazardous chemical mixtures by acute, chronic and genotoxic damage, estimating responses ranging from mortality to sublethal effects. The range of the results for mutagenicity, chronic and acute toxicity obtained in this last study has shown the importance of performing an integrated series both of genetic, and toxicity tests to assess environmental risk areas, like the data shown by Vargas et al. (2008) in the Caí River basin in the influence area of the petrochemical complex analyzed in the present study (Vargas et al., 2008).

Since contaminants may affect human health and biota, aquatic ecosystems should be studied. Assays that detect genotoxic potential should be carefully considered when evaluating xenobiotic agents in environmental samples because of their association with carcinogenicity, premature aging and degenerative diseases. Such assays have been have been useful to diagnose the environmental quality, and can indicate possible risks to human health and to the organisms that constitute the ecosystem (De Flora et al., 1993; Vargas et al., 1993; Lemos and Erdtmann, 2000; Vargas et al., 2008).

Acute toxic tests measure the drastic effects on organisms. Since they are relatively fast and low cost they are widely used to evaluate industrial effluentFs. Among the different animals used in acute toxicity tests, fish such as *Danio rerio* and *Pimephales promelas* have been systematically utilized and recommended, because they are at the end of the aquatic food chain and are sensitive to any changes in the environment (Bertoletti et al., 1989; USEPA, 1991; DIN 38412, 1989; APHA, 1995; Domingues and Bertoletti, 2006). Besides death, changes such as lethargy, loss of orientation and others can be detected in these organisms.

Chronic tests expose very young organisms up to the final phase of the ontogenic cycle, covering the reproductive phase, a period when individuals are more sensitive to environmental change. Although this modality requires more time to supply an answer to the acute tests, the results obtained are closer to reality.

The aquatic microcrustacean *Daphnia magna*, which is widely utilized in standardized tests for ecotoxicological studies (LeBlanc, 1980, 1982, 1985; Winner 1981; Day and Kaushik, 1987; Kukkonen and Oikari, 1992; Terra and Feiden, 2003; Terra et al., 2006), can be used to perform chronic tests. Furthermore, small cladocera grow fast during the ontogenic cycle (Romanowsky, 1984) and present protective mechanisms for the species, such as ephippia formation, which makes it easier to obtain quick answers in stressful environments.

These assays are appropriate to evaluate water samples, including industrial effluents. Moreover, it is important to assess sediment quality using these bioassays, because, besides showing the influence on the organisms exposed, the effect on the organisms that feed on these sediments can be inferred. Sediment, like water, is a spatially and temporarily variable source, but it has the capacity of accumulating persistent substances, and it has a greater probability of detecting a positive response (Terra et al, 2008). In recent years, further additional methods have been created to assess contaminants in sediments, since these are potential sources of water contamination (Terra and Schäfer, 2000).

According to the sequential ecotoxicological analysis methodology adopted in this study, genotoxicity was found at the sites where no acute or chronic toxicity was characterized. For this first experience in applying the model, model, mutagenic activity was analyzed using the *Salmonella*/ microsome assay. The assay is usually able to detect potentially mutagenic effects of chemical industrial waste mixtures (Houck, 1992; Claxton et al., 1995). Previous studies (Vargas et al., 1988) identified positive mutagenic activity in water samples from rainfall and industrial liquid effluents from the petrochemical industries, both for direct assays and after S9 fraction for access liver metabolites. This assay also proved significantly sensitive to investigate the presence of organic compounds originating from the petrochemical industry, in the area of environmental influence at different study periods (Vargas et al., 1993, 1995; FEPAM/PADCT/FINEP, 1997, 2004; Horn et al., 2004; Vargas et al., 2008).

The purpose of this study was to evaluate acute and chronic toxicity besides the toxicity found in samples of the last stabilization lagoon (L8) in the Integrated Liquid Effluent Treatment System and in the accumulation and safety drainage basins (B3, B4 and B7) using organisms with different trophic levels.

2. Material and Methods

2.1. Sampling sites

This study was performed in a petrochemical complex treatment area in Rio Grande do Sul, Brazil. The area received rainfall runoff, and different organic and inorganic effluents. The industrial area comprises a raw materials

center and eight second generation industries. The raw materials center produces 3 million t.year^{-1} of petrochemical compounds such as ethene, propane, butadiene, benzene, toluene, xylene and butene-1.

The rainfall effluents that do not suffer contamination resulting from the rainfall runoff and washing non-process areas, and some wastes below the treatment system level, are conducted to the drainage basins and storm canals which overflow into the surrounding area. Three accumulation and safety drainage basins (B3, B4 and B7) were sampled (Figure 1). B3 receives drainage waters from storm sewers and is located close to the coal storage area; B4 receives storm drainage water from the area close to the ash basin; B7 is intended to preserve the natural flora and fauna in the area, and it was planned to receive only the drainage from uncontaminated storm sewers in the adjacent area.

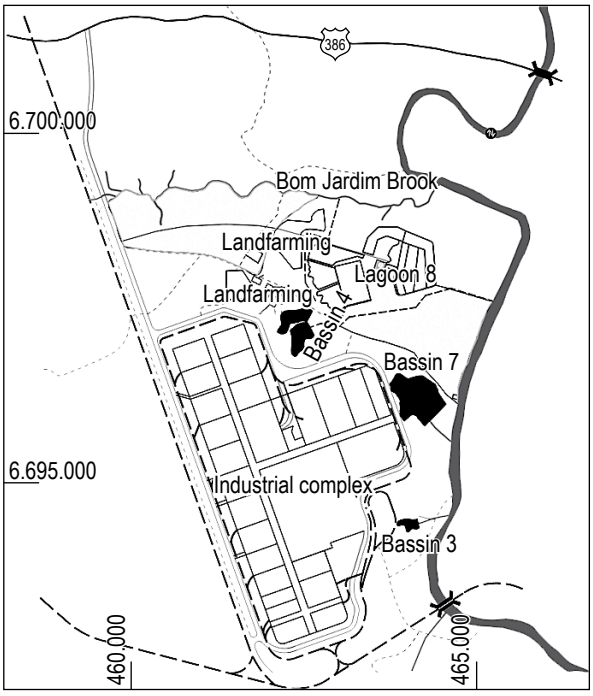

Figure 1. Sampling points of the study area.

Inorganic effluents are initially treated at the respective industrial plants and then conducted, to an organic basin where they will be treated together. The third treatment phase comprises eight interconnected lagoons. The last stabilization lagoon (L8) in the Integrated Liquid Effluent Treatment System that receives the industrial waste of this complex was also sampled. After this treatment phase, the effluents were spread on the soil. The samples were collected for different stages, diagnostic approaches and periods, from August/94 to April/04 (Table 1).

2.2. Ecotoxicological diagnosis

2.2.1. Acute assays (fish)

Acute assays using *D. rerio* and *P. promelas* were developed in the industrial area, B3, B7 and L8 between August/94 and January/96. The B4 basin was not evaluated during this stage of the study because its water column is usually insufficient to collect samples in a continuous flow system. Initially only *D. rerio* was exposed, according to a standardized methodology (ISO 7346 1996), and from March/95 onwards assays were also performed with young *P. promelas*.

The tests were performed with semi-continuous flow. The test-substance and the dilution water converged every 60 minutes from the source, to the system, and this in turn distributed the solutions to the mixers and later to the test-containers. This phase of the work was performed in a mobile laboratory controlled by a pre-programmed electronic system.

Up to the time of the test the fishes were maintained on special dehydrated fish food, frozen *Artemia* biomass and live nauplii of *Artemia salina*. The fish were kept in reconstituted water with a hardness equivalent to 40 mg.L^{-1} of $CaCO_3$ and constant aeration. The exemplars of *D. rerio* belonged to homogeneous lots and had a total length of 3.0 + 0.5 cm, and those of *P. promelas* were less than 60 days old. The organisms were selected randomly, placed and transported in two small 30 L barrels with reconstituted water. Each test exposed three specimens of *D. rerio* and five of *P. promelas*, in duplicate in different aquaria, with a

Table 1. Stages of the study including different diagnostic approaches, periods, samples and sites.

Stages	Periods	Samples	Sites
Acute toxicity (fish)	August/94 to January/96	Water	B3, B7, L8
Physicochemical parameters	August/94 to January/96	Water	B3, B7, L8
Chronic toxicity (*D.magna*)	April/95 to March/96	Water	B3, B7, B4, L8
	May/98	Sediments	B7
	November/00	Sediments	B3, B7, L8
Mutagenic and cytotoxic activity	June/93	Water	L8
(*Salmonella* /microsome assay)	November/96 and April/97	Water	L8
	June and November/03	Water	L8
	February and April/04	Water	L8

sample and negative control, for 48 hours. Beakers with a nominal value of three liters containing two liters of sample were used. The animals were not fed during the test. The negative control received reconstituted water (160 µS.cm^{-1} with a hardness of 40 mg.L^{-1} of $CaCO_3$) in a tank containing 250 L, previously aerated for 24 hours.

During the tests, the values of dissolved oxygen, pH and sample temperature were checked. The values of the first parameter at the three sampling sites were always within the range of test acceptability, ≥4mg.L^{-1}, despite the fact that the pH and the temperature were sometimes off standard (7.0 ± 0.5; 22.0 ± 2 °C) according to ISO 7346-1 (1996). Assays were performed to evaluate the toxicity of the samples in natura and diluted. Toxicity was determined by mortality. The LC50 48 hours for *D. rerio* (9 tests) and *P. promelas* (2 tests), using Potassium Dichromate as the substance of reference, was between 121.06 and 207.88 mg.L^{-1} (171.55 ± 26.55) and 136.93 and 183.28 mg.L^{-1} (160.11 ± 32.77), respectively.

2.2.2. Traditional chemical parameters

During these campaigns, the physicochemical characterization of the raw samples was performed according to APHA (1992), and the parameters evaluated were pH, sulphide, aluminum, iron, manganese, phenol, phosphate, mercury, dissolved organic carbon (DOC), oils and greases. The samples were collected on the mean dates of the exposure periods at every site studied.

2.2.3. Chronic assays (microcrustaceans)

Three monthly collections were performed, since simple water samplings are point collections showing only the moment when collection occurs. In order to perform chronic assays, samples of water were kept frozen in aliquots that were sufficient for daily use in order to prevent changes due to evaporation, the reaction of compounds and substances with each other, and the metabolic action of organisms.

Initially each assay exposed 18 *Daphnia magna* of Clone A, 2 to 26 hours old, in three beakers with a nominal value of 50 mL, containing 40 mL of sample per sampling point (270 individuals). The tests were developed in a germinator programmed for 16light/8dark and 21 ± 2 °C, for 30 days, ensuring exposure in the young, reproductive and senile stages. On alternate days, the organisms were transferred to a beaker with fresh medium, avoiding the accumulation of metabolites, overpopulation, fighting for space, food and dissolved oxygen.

Between 1998 and 2000, sediment was sampled to evaluate its influence on *Daphnia magna*. The samples were maintained at 4 °C, from collection to time of use. In order to perform the tests, the ratio was 1:4 sediment to culture medium M4 (v:v) (Terra et al. 2006). During the observations M4 was substituted on alternate days, while the sediment remained the same from beginning to end of the observations.

Culture sensitivity was evaluated fortnightly with potassium dichromate. Lots with LC50-24 hours (≅1 mg.L^{-1}) were accepted. The individuals were fed *Scenedesmus subspicatus* (10^7 cells.cm^{-3}) microalgae ad libitum. Cultivation was performed based on axenic unialgal cultures, in a CHU nutrient medium.

On alternate days, the individuals were counted, and births, deaths and ephippia recorded. The presence of ephippia was evaluated because these forms indicate that the system is not healthy. Under adverse conditions such as change in environmental quality, high population density, low food availability, and accumulation of metabolites, males and ephippia may be formed (Antunes et al., 2003).

Each biological parameter received indexes from zero to three, with a view to establishing a degree of damage between stations, as described below: I) Ephippia, all values below the maximum index found in the control were defined as zero, index three being most different from the control; II) Mortality, the values were considered normal within the probability of mortality in the control (20%), index three being the most distant from the control. Mortality in the control was never higher than 20%; III) Birth, a value that differed by up to 20% from the control was defined as normal. Since this is the accepted percentage for mortality in chronic tests, we consider it appropriate to extend it to evaluate births. In order to assign the index to this event it was only considered whether the percentage was 20% different from the control, independent of whether it was higher or lower. This procedure was chosen, because both the overpopulation of a species and its elimination triggered misadjustments in the biota due to the rupture of the preferential bonds in the trophic chain, besides competition for food by species different from those already integrated in the system. Classification by index shows the extent to which the environment is compromised, index three showing the most inappropriate conditions to maintain the biota, while index zero indicates the less polluted samples.

The analysis of ephippia and neonates to detect the difference between the sampling stations was performed using ANOVA and the Duncan Test.

2.2.4. Mutagenicity test: salmonella/microsome assay

For this assay the samplings were performed at L8 according to APHA (1992), with the modifications described in Vargas et al. (1995). The samples were divided into aliquots and maintained at –20 °C. The pre-incubation procedure (Maron and Ames, 1983) accompanied by the cellular viability test to control the cytotoxic potential of these samples (Vargas et al., 1988, 1993) was used to perform the mutagenic evaluation assay. The *Salmonella typhimurium* strains TA98 and TA97a, which detect frameshift mutation,

TA100 and TA1535, which detect base pair substitution mutation, were used. Water samples in increasing volumes (1, 1.5 and 2 mL) per plate, were incubated with 100 µl of tester bacterial cultures (1-2 X109 cells.mL^{-1}) in the presence or absence of S9 mix for 25 minutes in the dark at 370C. 1-3 mL soft agar, containing different agar and salt concentrations as described in Vargas et al. (1988; 1995). The S9 mix was prepared according to Maron and Ames (1983). The S9 fraction was acquired from Moltox SA, prepared from livers of Sprague-Dawley rats pretreated with a polychlorinated biphenyl mixture (Aroclor 1254).

The Salmonel program (Myers et al., 1991) was applied to evaluate the dose-response curve. The final criterion used took into account the reversion value equal to or more than twice the spontaneous one (Maron and Ames, 1983) and a reproducible dose-response curve with a significantly positive slope ($p < or = 0.05$). When only one of these criteria was met, the sample was considered indicative of mutagenicity. The sample was cytotoxic when cell survival was less than 60% as compared to the negative control (Vargas et al., 1993).

3. Results

3.1. First stage: acute toxic assays and physicochemical parameters

Forty-six acute toxicity tests using fish (*D. rerio* and *P. promelas*) were performed and presented negative responses, 19 with samples from LE8, 14 from B7 and 13 from B3.

Although the acute toxic responses were negative at B3, B7 and L8, it was found that in a few months the values of the physicochemical parameters at these sites were not within the permissible values according to Brazilian legislation on freshwater (Brasil, 2005) for B3 and B7 (Table 2) or the state administrative ruling that establishes the criteria and standards of liquid effluent discharge from this

Table 2. Comparative analysis of traditional chemical parameters results and legislation standards

Parameter (mg/L) [a]	Standards [b,c]	Site B3		Site B7		L8	
		Sampling	Value	Sampling	Value	Sampling	Value
pH	6 to 9 [b,c]	July/95	5.5	-	-	November/95	12.2
Sulphide	0.002 [b]	March/95	0.03	December/94	0.03	-	-
-	-	-	-	May/95	0.03	-	-
Aluminum	0.1 [b]	May/95	0.157	December/94	0.338	-	-
-	-	July/95	0.171	March/95	0.22	-	-
-	-	-	-	May/95	0.171	-	-
-	-	-	-	July/95	1.42	-	-
-	-	-	-	November/95	0.3	-	-
Iron	0.3 [b]	May/95	0.497	December/94	0.827	-	-
-	-	-	-	March/95	0.352	-	-
-	-	-	-	May/95	0.374	-	-
Manganese	0.1 [b]	May/95	0.14	December/94	0.396	-	-
-	-	January/96	0.117	March/95	0.19	-	-
-	-	-	-	May/95	0.178	-	-
-	-	-	-	July/95	0.147	-	-
-	-	-	-	November/95	0.344	-	-
-	-	-	-	January/96	0.521	-	-
Phenol	0.003 [b]	-	-	December/94	0.081	-	-
-	-	-	-	May/95	0.008	-	-
Phosphate	0.020 [b]	-	-	December/94	0.108	-	-
Mercury	0.0002 [b]	-	-	May/95	0.24	-	-
DOC	100 [c]	-	-	-	-	August/94	112
-	-	-	-	-	-	October/94	125
-	-	-	-	-	-	November/94	124
-	-	-	-	-	-	November/95	110
-	-	-	-	-	-	January/96	114
Oils/greases	10 [c]	-	-	-	-	August/94	14
-	-	-	-	-	-	November/94	11.3
-	-	-	-	-	-	May/95	11.2

[a]mg/L off pH

[b]class 2 river (Brasil, 2005)

[c]L8 (Rio Grande do Sul, 1995).

[d]DOC Dissolved organic carbon

petrochemical complex, for L8, (Rio Grande do Sul, 1995). At B7 during six months of samplings, changes could be observed in one to six of the parameters analyzed, and for L8, the parameter DOC presented five values above those defined by standard legislation.

3.2. Second stage: chronic toxic assays

Analyzing the number of ephippia, it was observed that B3, B4 and B7 are different from L8 ($\alpha = 0.05$), when the Duncan test is applied. More ephippia (181) were formed at B3 than at B4 (120), B7 (120) and L8 (21).

The frequencies of mortality in the control and at L8 were similar, whereas they were higher at the other sampling stations (Figure 2). It should be mentioned that many of the individuals exposed to samples from B4 and L8 became paler.

The number of births indicated that while 8,111 individuals were born to the controls, 5,891; 5,146; 5,850 and 13,873 individuals, respectively, were generated at B3, B4, B7 and L8. In order to evaluate the births, the difference between the control and each sampling station was calculated (Table 3). Greater hardness was observed at L8, as compared with the other stations. While in Controls the hardness was 240 mg.L^{-1} of $CaCO_3$, at L8 it varied from 90 to 100.

The indexes assigned to parameters ephippia, mortality and birth are presented in Table 4. Although L8 presented index zero for ephippia formation, no marked reproductive increment was observed (Figure 3). In Figure 3, the more distant the values are from the center of the axes, the greater is environmental change for the parameter considered. Hence, we can say that the most sensitive parameter to identify the level of environmental degradation is reproduction, since it was most distant from the center at all sites analyzed. The sum of the indexes corresponding to the three parameters per sampling station showed a small variation between the results of sites: B3 (30); B7 (29); B4 (28) and L8 (28).

Table 3. Monthly difference in births between the control and the sampling stations (*D.magna*).

Month	Site			
	B3	B4	B7	L8
April/95	+46	–44	+49	+853
May	–263	–273	–275	+307
June	–247	–177	–180	+229
July	–68	–122	–90	+680
August	+29	–133	–99	+57
September	–168	–170	–212	+54
October	–89	–26	–80	+421
November	–336	–269	–209	+134
December	–324	–230	–117	+252
January/96	–107	–191	–113	+478
February	–46	–208	–207	+519
March	–252	–341	–171	+646

(+) Reproduction greater than control.

(–) Reproduction lower than control.

Table 4. Indexes of ephippia, mortality and births per sampling stations. The higher the index the greater the change in the site.

Month	Parameters / Site											
	Ephippia				Dead				Birth			
	B3	B4	B7	L8	B3	B4	B7	L8	B3	B4	B7	L8
April/95	1	0	1	0	0	0	0	0	0	0	0	3
May	1	0	0	0	0	0	0	0	2	2	2	2
June	1	1	0	0	3	3	3	3	2	1	1	1
July	0	0	0	0	0	0	0	0	1	1	1	3
August	0	0	0	0	0	0	0	0	1	2	2	0
September	1	1	1	0	0	0	0	0	2	2	2	0
October	1	0	0	0	0	0	0	0	1	0	1	3
November	0	0	0	0	0	0	0	0	3	2	1	1
December	1	0	0	0	0	0	1	0	3	2	1	3
January/96	0	0	0	0	1	3	3	0	1	1	1	3
February	0	0	0	0	0	3	3	0	1	2	2	3
March	0	1	0	0	1	0	3	0	2	1	0	3
Total	6	3	2	0	5	9	13	3	19	16	14	25

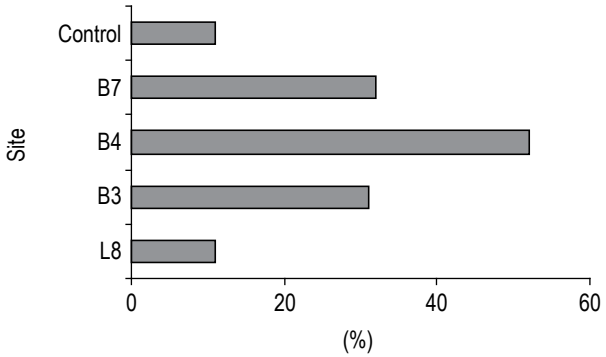

Figure 2. Mortality frequency (*D. magna*) above 20% per sampling station between April/95 and March/96.

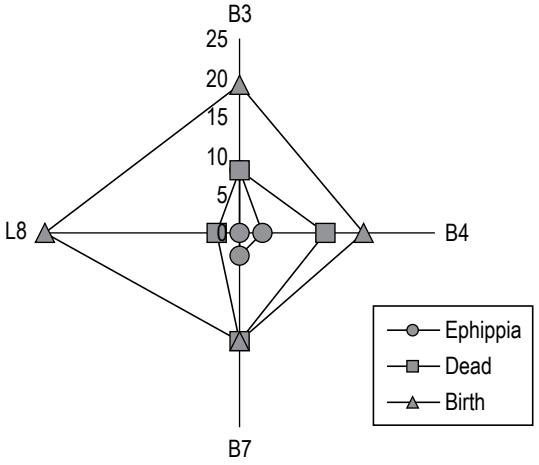

Figure 3. Sum of the indexes obtained per biological parameter between April/95 and March/96.

Sediment sampling performed in May 98 at B7 showed 100% survival among the exposed cladoceran, indicating absence of acute toxicity.

The same sampling, however, characterized chronic toxicity due to the diminished number of births. While in the control group 1,432 individuals were generated, making up 70% of the total offspring generated in the experiment, at B7 only 613 microcrustaceans were born, corresponding to 30%.

In November 2000, a new sediment sampling performed at B3, B7 and L8 indicated acute toxicity at B3 with 40% deaths on the sixth day of observations, another 30% on the eighth day, 20% on the tenth, reaching 100% mortality on the thirteenth day. At L8 30% mortality was found among the organisms exposed, while at B7 there was only a single death, within what was expected for this type of assay (20%). On evaluating neonate generation in these samples, we observed that L8 (393 neonates) and B7 (527 neonates) presented lower values in the generation of individuals compared to the control group (1409 neonates).

3.3. Third stage: mutagenic and cytotoxic activity

The mutagenic and cytotoxic events were observed at L8 in three samples analyzed at different periods studied during seven different samplings (A- June/93; B- November/96 and C-April/97; D- June and E- November/03, F- February and G- April/04). The results (Table 5) show a positive response for frameshift and base pair substitution mutagenic activity in the presence of metabolic activation in sampling A, strain TA98 (0.43 revertants.mL^{-1} of sample) and in sampling C, strain TA1535 (8.5 revertants.mL^{-1}), respectively. In the latter sample signs of mutagenesis by frameshift mutation were seen in strain TA97a, in the absence of S9 fraction (16.6 revertants.mL^{-1}). In the periods from June/03 to April/04 a seasonal study showed mutagenic activity only in winter (sample D) also by frameshift-type with S9 fraction.

Cytotoxicity was constant in samplings A and B with 55% rates of survival in direct assays in sampling A and 42% in the presence of S9 mix in both cytotoxic samplings (data not shown).

4. Discussion

This study, by acting on the organisms exposed, made it possible to define the presence of compounds with chronic or mutagenic effects in the storm drainage basins and in the last stabilization lagoon that receives the treated industrial liquid wastes.

An ecotoxicological assessment model was applied to these areas sequentially and selectively from the petrochemical complex source. In the first stage acute toxicity tests with *Danio rerio* and *Pimephales promelas* were used, accompanied by physicochemical parameters. Although the acute toxic responses were negative at B3 and B7, it was found that, during given months (Table 2), the values of the physicochemical parameters at these sites were not within the permissible values, mainly at B7 (Brasil, 2005). According to current law for storm basins, the water must present the same class of quality (class 2) adopted in the river basin which is influenced by the complex. This class of river should preserve the public water supply, irrigation, watering animals, primary contact recreation, artisanal fishing, navigation and protection of aquatic communities. At L8, some values of the physicochemical parameters (pH, DOC and oils/greases) were not within the permissible values (Rio Grande do Sul 1995). In an acute toxicity test, in September/95 and January/96, one exemplar of *D. rerio* died. Considering all the assays performed with *D. rerio*, these deaths represent 2% of the total number of exemplars exposed. In parallel tests with *P. promelas*, no mortality occurred in this species.

At the second level of evaluation, the presence of chronic effects in water samples at B3, B4 and B7 was characterized, and a change in the generation of individuals at L8. In this lagoon an intensive reproductive process began, resulting in overpopulation. The explosion of births observed at L8

Table 5. Samples of the last stabilization lagoon for liquid effluents (L8) with positive mutagenic activity expressed in revertants.ml^{-1}.

Periods	Samplings	Frameshift mutation		Base-pair substitution mutation	
		– S9	+S9	–S9	+S9
June/93	A	-	0.43 ± 0.02 (TA98)	-	-
November/96	B	-	-	-	-
April/97	C	16.6 ± 1.13 (TA97)	-	-	8,5 ± 1,03 (TA1535)
June/03	E		21.5 ± 1.89 (TA98)	-	-
November/03	F	-	-	-	-
February/04	G	-	-	-	-
April/04	H	-	-	-	-

Revertants per milliliter of water ± S.E.M. using Salmonel software (Myers et al., 1991); – S9 absence and + S9 presence of metabolic activation; – negative response; TA98 and TA97 frameshift mutation strains; TA1535 base-pair substitution mutation strain; Negative Control in revertants/plate: 25 ± 0.5 (TA98-S9), 30 ± 0.7 (TA98 + S9), 122 ± 24.4 (97a – S9), 10 ± 2.1 (TA1535 + S9); Positive Control in revertants/plate: 4NQO (0.5 µg.plate^{-1}) 258 ± 44,1 (TA98-S9), 823 ± 83.9 (TA97a –S9); 2AF (10 µg.plate^{-1}) 488 ± 31.9 (TA98 + S9); 1370,3 ± 415,21 (1535 + S9).

may have been triggered by the presence of Phosphorus and Nitrogen in this lagoon. Analyzing the ephippia it was observed that a more significant number of them was formed in the storm basins (B3, B4, B7) than in treated effluent (L8).

A similar response was observed in the frequencies of mortality among the control group and L8, differing from the other sites where these frequencies were high, showing the action of the environment on the microcrustaceans.

The number of births indicated that the sites influenced the development of the crustaceans. There was a 27, 37 and 28% reduction in the number of births at B3, B4, B7, respectively, showing chronic toxicity. On the other hand, at L8, a 71% elevation in the number of births was observed indicating the existence of factors that although not toxic compromised the environment. The zooplankton community of the stabilization lagoons in the tertiary system (L8) of this industrial complex is typical of environments that have undergone eutrophication. *Moina micrura* and *Moina macrocopa americana*, which are species associated with eutrophic characteristics, are found in these lagoons. There is also an increase in the number of rotifera towards L8, a lagoon that presented maximum densities of organisms indicating a eutrophic environment (Bohrer, 1995). The growth of *Daphnia magna*, higher than in the control group, is probably due to the high concentration of nutrients, agreeing with the information mentioned. Although hardness was high at L8 as compared with the other stations, this does not account for the overpopulation, since the latter was even greater than the control. According to Terra and Feiden (2003), the variation in hardness does not alter the reproductive activity of the species.

The indexes assigned to parameters ephippia, mortality and birth (Table 4) show that birth was the most sensitive biological parameter, followed by mortality and, lastly, by ephippia formation. Although L8 presented index zero for ephippia formation, the overpopulation that occurred suggests a change in environmental quality (Figure 3).

The sum of the indexes corresponding to the three parameters per sampling station showed that the substances present in the environment influence the development of the cladocerans. This flow method of waste disposal, common in petrochemical areas, may endanger local fauna (Schroder et al., 2000). It is observed that B3 presented 30 as the sum of indexes identifying the site as the one with the worst quality. This site was followed by B7 with the sum 29, B4 and L8, both with 28.

While the observations were performed, a difference was found in the values of hardness ($CaCO_3$) among the four stations evaluated. A previous paper showed that *D. magna* cultivated at a mean hardness of 300 $mg.L^{-1}$ $CaCO_3$ were not influenced in their development when exposed to values of 50 $mg.L^{-1}$ $CaCO_3$ (Lewis and Maki,1981). This information is reinforced in chronic assays with hardness values between 10 and 250 $mg.L^{-1}$ $CaCO_3$, which showed that there was no significant difference in survival and reproduction among the extreme values (Terra and Feiden, 2003).

The sediment samples collected in May/98 showed that at B7, although survival was 100%, there was a 57% reduction in the births, characterizing the total absence of acute toxicity and the presence of sediment samples collected in May/98 showed that at B7, although survival was 100%, there was a 57% reduction in births, characterizing the absence of acute toxicity and presence of chronic toxicity.

In November 2000, new sediment samplings at B3, B7 and L8 indicated acute toxicity at B3 with 100% mortality at the end of the observations. In this cycle of exposure of microcrustaceans, chronic action was found both in the B7 and the L8 samples, with a reproductive decrease of 63% and 72% compared to the control. The microcrustacean *D. magna* has shown good responses in sediment exposure tests (Nebecker et al., 1986, 1988; Suedel et al., 1996; Terra et al., 2006; Gillis et al., 2005). Accumulated pollutants, such as PAHs (Ankley et al., 1996; Ireland et al., 1996; Gewurtz et al. 2000) are deposited in the sediment and later they or their by-products are released in living organisms (Terra et al., 2006).

In the last stage of this study the responses show the persistence of mutagenic potential of the treated effluent, promoting the induction of different types of damage to the DNA, both for direct assays and for metabolites generated by biotransformation in the liver, during the different study periods. Mutagenic activity responses in a *Salmonella*/ microsome test were previously found at B3, B4, B7 and L8 (Vargas et al. 1988). Ames tests performed between 1984 and 1991 at L8 presented a mutagenic effect in 41.7% and signs of mutagenicity in 8.3% of the analyses performed (Noll et al., 1994).

Chemical tests performed in July 1997 (unpublished data) identified some concentrations of dioxins and furans in B3, B7 and L8 water and sediment samples. In November 2000, organic compounds such as fluorine (B3, B7), fluorene (L8) and pyrene (B7, L8), naphtalene, crysene and fluoranthene (L8), besides low concentrations of dioxins and furans (B3, B7), were identified in the sediment samples.

In the sequential evaluation methodology proposed in this study the use of sets of biological assays enabled the characterization of different levels of contamination during the treatment stages investigated. Thus, it could be diagnosed that, although there is no acute toxicity in the storm water accumulation basins, the latter are compromised and may cause chronic toxic effects. It could also be diagnosed that, after passing through the treatment lagoons, the final effluent presents substances with mutagenic properties. Chronic toxicity was present in L8 sediment samples during the last study period showing the deposition of hazardous persistent compounds or compounds generated during the treatment of liquid effluents.

After this treatment phase, eight interconnected stabilization lagoons, the fact that these effluents were sprayed onto the soil could explain the presence of toxic and mutagenic substances (Vargas et al., 1993, 1995; FEPAM/ PADCT/FINEP, 1997, 2004; Horn et al., 2004; Terra et al., 2006; Vargas et al., 2008), affecting the quality of the Caí river basin in the area under the influence of this complex as already stated before (Terra and Feiden, 2008). This study was an ecotoxicological assessment model to develop new legislation in Rio Grande do Sul, Brazil (Rio Grande do Sul, 2007).

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