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Research Article

Highlighting Genotoxic, Teratogenic and Toxic Effects as Biomarkers for River Quality in a Petrochemical Industry Area

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Abstract

Petrochemical industry wastes are anthropic contributions that release compounds with a toxic and/or mutagenic potential to alter the ecosystem dynamic and population health into the environment. Water and sediment from 1988 to 2006 were investigated at four sites from the source to the mouth of Bom Jardim Stream, one of the streams forming the Caí River Basin, Southern Brazil. It is adjacent to the deposition site of treated effluents on soil in the Petrochemical Complex. In the tests performed with raw water, mutagenic activity was evaluated using the Salmonella/micro some assay and significant responses were detected at all sites, with a gradual elevation towards the stream mouth, where 100% of positive responses were found he most frequent event was the cytotoxic effect in 60% of the samples from the source and 100% from the other sites. A high percentage of malformations and toxicity was found with *Danio rerio* embryos test at the point with the highest frequency of mutagenic responses. The sediment samples showed benzene during one of the sampling periods. Studies on pesticide bioaccumulation in the *Gymnogeophagus gymnogenys* species did not show results explaining the biological effects observed in this area. These data show that in Salmonella/microsome assays, Embryo test with *Danio rerio* and a chronic toxicity analysis with D. magna were sensitive for the diagnosis of environmental quality degradation and are appropriate for environmental diagnosis in regions impacted by the petrochemical industry.

Keywords: *D. magna*; River water; *Salmonella*/microsome assay; Sediment; *Zebrafish embryo (Danio rerio)*

Introduction

Studies evaluating environmental quality indicate that anthropic influence is modifying ecosystems, as more contaminants of different kinds, which can interfere significantly in the trophic chain structure, are released. Some of these compounds substances our agents are bio-accumulators and can interfere in the energy and nutrient flows of the biological chain and Petroleum compounds from represent an important source of environmental pollution. An effective biological early warning system for the detection of water contamination should employ tests *in vivo* and *in vitro* and species that rapidly react to the presence of contaminants in their environment [1-3].

After several environmental contamination incidents, many researchers have reported the importance of further investigation regarding possible consequences to the environment [4-6], emphasizing that when these substances are released over time; they are highly relevant for organisms in general from the toxicological standpoint for organisms in general. The environmental stressors may affect the entire process with different responses for each species and individual [7]. Several authors have recommended using different organisms, including fish or other aquatic organisms, to evaluate the carcinogenic, Genotoxic potential in environmental biomonitoring [8-10]. These studies have shown that exposure to Genotoxic agents, besides damaging the DNA, may be associated with diminished growth and development of aquatic organisms, and decreased survival of embryos and adults [11]. Contaminants from the petrochemical industry are persistent and highly toxic to the ecosystem [12], besides being difficult to degrade, harming the biota and causing an imbalance in the ecosystems. Under given circumstances they may be a relevant risk for human populations [13,14]. Early detection of dangerous exposure using Genotoxicity biomarkers may significantly reduce the occurrence of the effects. The mutagenic potential of environmental samples has been characterized as one of the early parameters to evaluate the presence of pollutants in different environmental compartments (15,16). Short-term assays have been used to screen the potential risk of complex mixtures of Genotoxicity. The use of biomarkers becomes increasingly important in exposure assessment since it provides more exact information on actual internal exposure (target dose) to an agent. Each bioassay has particular strengths and weaknesses, in an integrated research strategy combining chemical/biotoxicological approaches for the evaluation of complex mixtures, different genotoxicity tests having different genetic end-points should be considered [6,17-20]. A specific application is the analysis of water resources covering surface and interstitial waters and the sediment matrix, by measuring their effect on the DNA [21-25].

Evaluations performed in industrial areas of Rio Grande do Sul, Brazil, have demonstrated the validity of the Salmonella/microsome assay to diagnose the presence of compounds with cytotoxic, mutagenic and potentially carcinogenic activity. The association of this assay, with tests that measure acute and chronic toxicity in aquatic organisms, for instance using *Daphnia Magna* has been essential, to define regions under the influence of sources of pollution [26-28] that are potentially impacted by organic pollutants [22,25,29,30] and heavy metals [31]. In these potentially impacted regions the mutagenic agents of different origins can interact, forming complex mixtures that are difficult to characterize chemically, where the most reactive compounds may be present at small concentrations. Thus, the definition of the possible chemical classes of the mixture helps identify the agents that potentially cause biological damage. This approach allows an advance in the management of preventive control measures, aiming to protect human health and the genetic patrimony of the populations [26].

In a literature review, [16] emphasized that applying the *Salmonella*/microsome assay to the study of water quality showed that some rivers around the world, especially in Europe, Asia and South America, are contaminated by mutagenic substances that have a direct and indirect action. Papers report that these rivers are potentially contaminated by untreated or partially treated wastes from chemical and petrochemical industries, refineries and oil spills, untreated domestic sewage and pesticides [16,19,32]. Many of these studies attempted to define the chemical compounds responsible for the mutagenicity and Genotoxicity of surface waters. Heavy metals, Polycyclic Aromatic Hydrocarbons (PAHs), heterocyclic amines, and pesticides were identified among these compounds. [25,30,33-35].

Studies showed evidence that the initial stages of fish life are potentially sensitive, and are a useful tool to look for possible toxic compounds. Currently the *Danio rerio* (Zebrafish) species has been used as a model for toxicological evaluation, in which the effect of contaminants on embryo development can be evaluated [36-39], supplying data related to mammalian models of exposure to environmental toxic agents [40]. Assays using zebrafish, in the initial phase of their development have been widely used due to several advantages, such as high fertility and quick development, easy visualization of their morphology in the embryonic phase and their sensitivity to the effects of the toxic compounds [41-43] besides evaluating different parameters of morphological, physiological and teratological effects abnormalities [42,44] Some studies have used the zebrafish embryo assay (*Danio rerio*) species, to monitor environmental quality, evaluating the sediment in lakes and rivers . [1,31,45-47].

This study presents a retrospective integrated evaluation of the quality of Bom Jardim Stream (the period from 1998 to 2005), one of the streams that from the Caí River Basin in Rio Grande do Sul, Brazil, analyzing samples of surface water, sediments and aquatic organisms collected from the source to the mouth. The stream drains the disposal area for effluents from a Petrochemical Complex. Previous studies, evaluating levels of toxicity and genotoxicity in the Caí River Basin, showed evidence of chronic and mutagenic toxicity responses at the confluence of the mouth of Bom Jardim Stream with the main river [22,25,28,48-52].

Material and Methods

Study area

Bom Jardim Stream, the area studied, is one of the sources that form the Caí River Basin in *Triunfo*, Rio Grande do Sul, Brazil (Figure 1). The climate of the region is subtropical, with uniform precipitation throughout the year. The region of Bom Jardim stream presents average temperatures oscillating between - 3 and 18ºC in the coldest months and in the warmer months exceeding 22ºC in the warmer months. The total rainfall of the region is about 1350 mm, with winter being the season with the highest amount of rainfall, while autumn and spring are less rainy [26,53].

Sampling sites

Four sampling points were chosen along this stream, located near a Petrochemical Complex in the state. Bom Jardim Stream is under the indirect influence of the industrial park, since, after primary, secondary and tertiary treatment the liquid effluents are sprayed on the soil close to this body of water [23,25,28,48] The second area of reference for samplings of *Gymnogeophagus gymnogenys* (family Cichlidae) is Fortaleza Lagoon located in the coastal town of Cidreira - RS. It is considered a Class 1 unpolluted area, used for domestic water supply after simplified treatment, irrigation of vegetables and fruits, primary contact recreation, and to protect aquatic communities [54].

The sampling sites were called by the initial letters of the watercourse, followed by the distance from the mouth (Kilometers), and thus the Bom Jardim Stream points were called: BJN (source; 29º51'8''S - 51º28'42''W), one of the sources that form this stream, located upstream from the area under the influence of the deposition of industrial effluents; BJ002 (29º50'27''S - 51º23'37''W) located in front of the area where the liquid effluents are sprayed; BJ001 (29º50'33''S - 51º24'96''W) upstream from the disposal area of the complex wastes; BJ000 (29º50'11''S - 51º21'01''W) located at the stream mouth, close to the Caí River. This site receives drainage water from the last liquid effluent disposal area and from the industrial waste treatment area with an indirect contribution from the petrochemical industry (Figure 1).

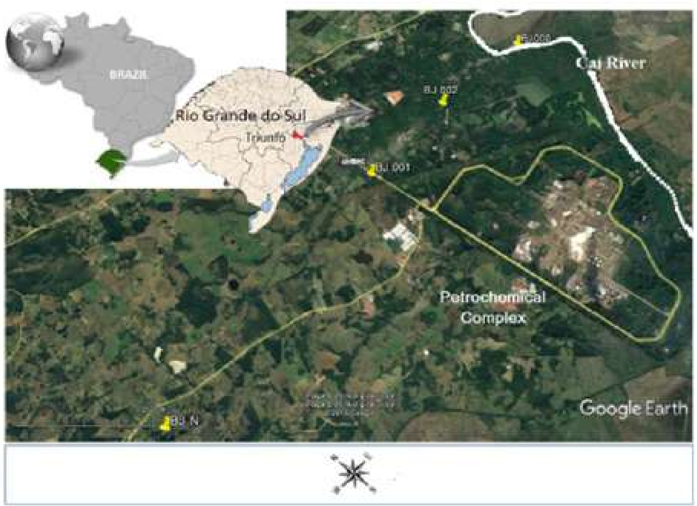

Figure 1: Sampling points of the Bom Jardim Stream in area under petrochemical influence (from the source BJN; BJ001; BJ002 to the mouth BJ000).

Sampling

The samples were collected between 1998 and 2005 with a total of 19 samplings. (Table 1) specifies sampling dates, compartments analyzed and assays used for the diagnosis. The collection of raw water samples followed the procedures standardized in APHA (1992; 1995); they were transported to the laboratory under refrigeration, stored at 4°C for up to four days, subdivided into aliquots and stored at - 20°C until the assay was performed. Sediment was collected by a grab sampler (Petersen), transported on ice to the laboratory, and stored in the dark at 4°C until it was tested [51,54-56] The glassware was carefully washed in order to eliminate any organic toxic or metal residue. Therefore, neutral liquid soap, nitric acid p.a. diluted at 50% and acetone p.a. were used.

Sites/ Year of sampling	Sampling	Season	Assay
BJ000/BJ001/	I	summer	b,c
/BJ002/BJN	II	summer	a,b,c
1998 - 2002	III	autumn	a,b,c
	IV	winter	a,b,c
	V	spring	a,b,c
	VI	summer	a,b,c
	VII	spring	b,c
	VIII	spring	a,b,c
	IX	spring	b,c
Fortaleza	X	winter	d
Lagoon	XI	spring	d
1999 - 2000	XII	summer	d
BJ000	XIV	summer	d
1999 - 2000	XV	autumn	d
	XVI	summer	d
	XVII	summer	d
	XVIII	winter	d
BJ000 2005	XIX	summer	e

Sites: BJN, BJ001, BJ002, BJ000, Fortaleza Lagoon.

Table 1: Site information according to sampling place, season, compartments analyzed and assays used.

Assays: a - *Salmonella*/microsome assay by direct concentration method - water; b - Chemical analysis- sediment; c - Chronic assay with Daphnia magna - sediment; d - Chemical analysis of pesticides - sediment and fish *(Gymnogeophagus gymnogenys)*; e - Embryo - Test *(Danio rerio)* - dry sediment.

The species *Gymnogeophagus gymnogenys* were collected at four distinct times in Fortaleza Lagoon, one at BJN and seven at the mouth of Bom Jardim Stream (BJ000) [20,57,58]. These species were chosen because, within the group sample, their size is adequate to collect samples of the liver and because they usually feed on the river substrate. The animals were captured with a seine net and a cast net, and the livers were removed immediately after biometry and sex determination, washed with ice-cold 1.15% KCl and stored in liquid nitrogen (-170°C), for up to 8 months. A representative pool for each point analyzed was prepared at the time of analysis.

Diagnostic approaches

Evaluation using the *Salmonella*/microsome assays

In order to evaluate different volumes of water samples in the Salmonella/microsome assay, a variation of the classical test for analysis of growing quantities of samples was used (1000, 1500 e 2000 µl/plate), sterilized with Millipore filters (0.22µm). The amounts of Agar and sodium chloride (NaCl) used in Agar were modified so that the final concentration per tube would remain identical [24,25]. To measure mutagenic activity, the Salmonella/microsome assay was used with the pre-incubation method in the presence and in the absence of a liver microsome fraction - fraction S9. This fraction was acquired from MOLTOX (Molecular Toxicology Inc, Boone, NC), prepared from *Sprague-Dawley* rats pre-treated with AROCLOR 1254 to which co-factors were added at the time of the assay - S9 mix fraction. To measure the two types of classic molecular mutations, strains TA100 and TA1535 were used for base pair substitution damage, and strains TA98 and TA97a, for frame shift mutation [2,59].

The dosages were performed in duplicate and when necessary the experiments were repeated to confirm the result. Negative (sterile distilled water) and positive controls (Sodium Azide - AZS, CASRN. 26628-22-8 Merck do Brasil; 4-nitroquinoleine oxide - 4NQO, CASRN. 56-57-5 e 2-aminofluorene - 2AF, CASRN. 153-78-6, Sigma, St. Louis, MO) were included in each assay. This study was complemented determining cytotoxic activity as described in [24]. The percentage of cell survival was calculated in relation to the number observed in the negative control of the test.

Positivity criteria: The mutagenesis response as calculated by analyzing the linear portion of the dose-response curve. The number of revertants per plate (rev/plate) was analyzed in the SALANAL program (SALANAL - Salmonella Assay Analysis version 1.0 of Research Triangle Institute, RTP, North Carolina, USA), selecting the regression models for the linear portion of the dose-response curve (Bernstein et al., 1982). The sample was considered mutagenic in the presence of positive significance in the regression analysis accompanied by a significant ANOVA $p \geq 0.05$. The sample was considered cytotoxic when the percentage of survival of the test organism was less than 60% of that observed in the negative control [24].

Correlation of the cytotoxic and mutagenic response with the physicochemical parameters

The correlations between the biological responses and the traditional physicochemical parameters were evaluated. These parameters were obtained from the monitoring data base created by FEPAM (Chemistry Division of the Department of Research and Laboratory Tests of FEPAM), following standardized methodologies (pH, temperature, conductivity, turbidity, dissolved oxygen, chemical oxygen demand, biochemical oxygen demand, chloride, suspended solids, dissolved solids) heavy metals (cadmium, iron, manganese, zinc, aluminum) and AOX [14] (Standard Methods for the Examination of Water and Wastewater, 1985).

The biological variables prioritized for statistical analysis were: Total Mutagenic Response (MUT) for the Salmonella/microsome assay in the absence and in the presence of the S9 metabolization fraction which correspond to acronyms MUT-S9 and MUT+S9, where the Mutagenesis Index corresponds to the mean of the values observed in the sample in relation to the values observed in the negative control, in assays with and without S9. The ranks were stipulated through the sum of the four strains used and correspond to: 0=absence of mutagenesis; 1= indicative response and 2= positive response for general mutagenic response (MUTG). MUTG is the sum of the ranks observed for the strains in the absence and in the presence of fraction S9, where a mutagenic response was observed in at least one of the conditions. For the toxicity (T) response in the absence and in the presence of S9 fraction (T-S9 and T+S9), the following scores were considered: 0: negative response and 2: positive response, and for the General Toxic response - (TG), the sum of ranks observed in the absence and presence of metabolization. Analyses were performed applying a Pearson correlation comparing traditional physicochemical parameters, heavy metals and AOX to the biological responses obtained in this study.

Chronic assay with *Daphnia magna* – sediment

A total of 80 D. magna (clone A) juveniles were exposed to sediment samples, per site sampled. Cultures and tests were kept in separate incubator chambers to minimize contamination. The daphnids used in the assays were obtained from cultures kept at a density of 25 adult individuals per 1000mL in M4 medium [60]. These culture conditions maintained the micro crustaceans in the parthenogenetic reproductive stage. All daphnids in this study were from the third or later brood. Before exposure to sediment, the organisms were submitted to tests for sensitivity to potassium dichromate, and homogeneous lots (LC50-24h 1,00mgK2Cr2O7 ± 0.05) were used. Eight 21-d semi-static tests were conducted in incubators at 20 ± 2ºC under a 16h light/8h dark photoperiod. The experiments began by adding ten newborn water fleas, disposed individually in 50 mL beakers for each period of the study. The beakers were covered with laboratory film to prevent medium evaporation or contamination. Each beaker received one part of sediment and four parts of liquid medium (v: v), in the same ratio used in other experiments [27,56].

The cladocerans were monitored on alternate days, for mortality and reproductive status, at which time neonates were counted and removed. After this procedure, M4 medium was introduced through the inner wall of the beaker, preventing a breakdown of sediment stability. The daphnids were fed on alternate days with a culture of green algae, *Desmodesmus subspicatus* (Chodat, 1926)

(0.7 ml; 107 cells.cm⁻³), a sufficient amount to ensure feeding until the next handling. An evaluation of the data defined the level of ecosystem alteration (acute or chronic). The total number of births supplied information regarding the level of chronic toxicity. The presence of acute toxicity was established through the percentage of survival, where the expected value was to be equal to or higher than 80%.

Analysis of organic compounds and organochlorinated pesticides in fish tissues and sediments

Samples of fish (*Gymnogeophagus gymnogenys species*) and of sediments were collected at different seasons of the year (Table 1), during a two-year period to evaluate the presence of organic compounds (sediments) and organochlorinated pesticides in fish. The following organic compounds were analyzed: benzene, chlorobenzene, 1-2 dichlorobenzene, 1-4 dichlorobenzene, ethyl benzene, toluene, m-xylene, o-xylene, p-xylene. The fish muscles were ground in a blender for the analysis of organo-chlorinated pesticides and then 40 g of anhydrous sodium sulfate were added to 20 g of the ground material. After 20 minutes, 100 mL of petroleum ether were added and the mixture was placed in an ultrasound with an ice bath for 5 min. The extract was filtered through glass wool and 20 g of anhydrous sodium sulfate, and the filtrate was collected.

The extractions were repeated with 100 mL and 70 mL of petroleum ether and the extracts were placed together again after filtration. Then a 25 mL aliquot of the extracts was concentrated at 3 mL using a rotary evaporator at 40ºC and transferred to a column containing 4 g of Florisil (10 X 300 mm).The elution of the Florisil column analytes was performed using three petroleum ether mixtures: ethylic ether, at the proportions of 6:94, 15:85 and 50:50 (v/v). Each of the eluates was concentrated in a rotatory evaporator and transferred to *Kuderna-Danish* tubes. The flask content was evaporated under nitrogen flux at 40ºC.

For chromatographic analysis the extracts were resuspended with 10 mL of internal standard, pentachloronitrobenzene (PCNB), at a concentration of 5 ppb. One microliter of this solution was injected into a Varian 3800 gas chromatograph equipped with an electron capture detector (63Ni) and DB 608 capillary column (30 m x 0.32 mm). The equipment operation conditions were defined as: injector temperature: 230°C; initial temperature of the column: 130ºC; final temperature of the column: 270ºC (6 ºC/min); detector temperature: 310ºC; injection volume: 1 mL (splitless, after 1 min. split 1:50); carrier gas flux (N2): 1 mL.min-1; make up gas flux (N2): 29 mL.min-1. This method is linear from 0.4 to 25.0 ppb for aldrin, α-BHC, ß-BHC, δ-BHC, ▲-BHC, heptachlor and epoxide heptachlor; from 0.8 to 50.0 ppb for 4,4'-DDE, dieldrin, endosulfan I, endosulfan II and endrin, and from 2.4 to 150.0 ppb for 4,4'-DDD, 4,4'-DDT, endosulfan sulfate and endrin aldehyde. Solvents in sediments were determined with 1 g of sample in a 22 mL flask containing 10 mL of Milli-Q water. Fifty micro liters of dry methanol were added, and then the flask was sealed and heated for 20 minutes at 85ºC. Then micro-extraction was performed in solid phase using 100 µm polydimethylxilone fiber (Supelco®).

The adsorption was performed for 3 minutes and desorption lasted 2 minutes, and was done in a Varian model 3300 gas chromatograph equipped with a DB 624 megabore column and flame ionization detector. The operation conditions of the equipment were defined as; injector temperature: 230 ºC; initial temperature of the column: 40 ºC; final temperature of the column: 265 ºC (5 ºC/min); detector temperature: 250 ºC; carrier gas flux (N2): 5 mL.min-1; make up gas flux (N2): 25 mL.min-1. The method limit of detection is 0.5 ppm.

Embryo-Test (*Danio rerio*) in sediment

Site BJ000 of Bom Jardim Stream was chosen to investigate sediment toxicity in the assay with *Danio rerio* embryos. Two hundred embryos were analyzed, exposed in samples of raw sediment, submitted to vacuum/cold drying and rehydrated with artificial water. The CHRIST-ALPH 2-4 LOC-1m was used to dry sediment at temperatures that vary from - 54Cº to 24Cº under an 800 mbar pressure for a 72-hour period. Different concentrations of dry sediment were evaluated for the assay: A - 3.0 g; B - 2.25 g; C- 1.50 g; D - 0.75 g. In all samples 25 mL of artificial water were added, lightly homogenized, and the sediment rehydrated. A control sample (artificial sediment, 3.0 g of quartz powder - plus 25 mL of artificial water) was used for all experiments (negative control). Artificial water was produced according to DIN 38415-6 (58.8 mg/L $CaCl_2$ x 2 H_2O, 24mg/L $mgSO_4$ x 7 H_2O, 12.6 mg/L $NaHCO_3$, 5.5mg/L KCL) stock solution was diluted 1:5 with double-distilled water. Zebrafish (*Danio rerio*) were maintained according to the Wester field M standard protocols [61].

After fertilization, the eggs were transferred to Petri dishes and inspected 12, 24, 36, 48, 72, 84 and 96 h after initiation of the exposure using an inverted microscope at 10X and 40X (Olympus); they were incubated in a heating cabinet and kept at a temperature of 26.0 ± 0.1ºC and at a 12:12h dark-light cycle. The endpoints were used to determine the mortality of the embryos and larvae in the embryo toxicity test with zebrafish during a total period of 96 hours and the embryos were removed daily. The embryos were evaluated during their development for endpoints: a- Lack of somite formation; b- Coagulation of embryos; c- Non-detachment of tail; d- Non–development of eyes; e- Developmental retardation; f - Lack of heart function; g- Lack of blood circulation, h- Lack of pigmentation, i- Edema formation, j- Lack of hatch, l- Spinal deformations (Scoliosis, Helical body), according to Nagel's criteria of lethality [62].

Statistical analysis

Graph Pad/Prism 5 was used to analyze the graphs and the

ANOVA test was used to compare the results; program SALANAL was used for mutagenicity assays; program SPSS/PC+ (version 8.0) was used to analyze the physicochemical parameters.

Results

The diagnosis performed at Bom Jardim Stream, which is under the indirect influence of the final disposal of petrochemical effluents, covers the investigation of the waters through the cytotoxic and mutagenic activities accompanied by traditional physicochemical parameters. The presence of organic compounds in this matrix and in fish muscles was investigated in order to evaluate sediment quality, besides biological assays to evaluate the chronic response in *Daphnia magna* and teratogenic effects using embryos of the *Danioa rerio* species as bioindicator.

Salmonella/microsome assay by direct concentration method

Cytotoxicity analysis

Four sampling points along Bom Jardim Stream were analyzed at six different periods (Table 1). The results showed cytotoxicity as the main effect present in 60% of the samples at site BJN and 100% for sites BJ001, BJ002 and BJ000, considering the information obtained in at least one of the treatments (±S9 mix). More frequent responses could be observed in the assays after metabolization.

In (Figure 2) (A) and (B) the results of cell survival can be visualized for each point sampled. The values below the 60% survival limit are considered cytotoxic. In Figure 2 (A), assays after S9 metabolization are presented, considering the response as positive due to the presence of cytotoxicity in at least one dose, and it was found that only BJN and BJ001 did not present cytotoxicity in sampling IV. In Figure 2 (B) the effect of cytotoxicity was less constant in the assays without S9 fraction mix. In general, the effect of cytotoxicity was constant at all sampling times at the different seasons of the year.

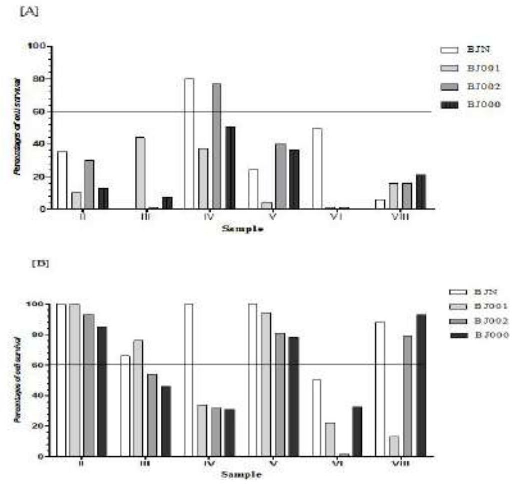

Figure 2: Cytotoxicity percentages of cell survival in the *Salmonella*/ microsome assay for samples of water from Bom Jardim Stream [A] in the presence of metabolic activation (S9) [B] in the absence of metabolic activation. Values below 60% (broken line) show cytotoxicity.

Mutagenicity of Water

Although the mutagenic effect is less frequent, it was observed in the different areas of Bom Jardim Stream. (Table 2) describes the positive mutagenesis values or those that are indicative in revertants/mL of the samples. The variations of the intensity of responses in revertants/mL for a base pair substitution range from 3.5 ± 1.41 (site BJ000, strain TA1535-S9) to 109.5 ± 53.27 (site BJ000, TA100-S9) and responses of the frame shift mutagens range from 1.9 ± 0.76 (BJ000, TA98-S9) to 159.5 ± 6.79 (BJN, TA98 + S9).

Considering TA97a, although the intensity and frequency of the responses were lower, values of 10.4 ± 2.37 could be detected for site BJ000 in the presence up to 33.5 ± 18.00 in the absence of S9. The positive and indicative responses were mostly the result of direct action (21 samples) the result of direct action rather than after metabolization (12 samples), of which 14 responses were for frame shift and 19 for base pair substitution damages.

Area/ Sampling		TA 98		TA 97[a]		TA 100		TA 1535	
		- S9	**- S9**	**- S9**	**- S9**	**- S9**	**- S9**	**- S9**	**- S9**
Summer II	**BJN**	2.9 ± 0.96				52.3 ± 22.19			
	BJ001	4.1 ± 1.20		33.5 ± 18.00					
	BJ002	3.4 ± 0.59		13.2 ± 3.32					
	BJ000				10.4 ± 2.37	WM			
Autumn III	**BJN**								
	BJ001								
	BJ002	2.7 ± 1.05							
	BJ000	2.9 ± 0.47				109.5±53.27		3.5 ± 1.41	
Winter IV	**BJN**						33.0 ± 13.99		
	BJ001					64.9 ± 12.29			
	BJ002					27.2 ± 6.83	33.8 ± 28.30		7.6 ± 3.63
	BJ000	4.2 ± 1.35				33.6 ± 12.54		WM	42.7 ± 7.50
Spring V	**BJN**		159.5 ± 6.79						
	BJ001								
	BJ002								
	BJ000	1.9 ± 0.76							16.0 ± 3.22
Summer VI	**BJN**	4.0 ± 1.99							
	BJ001					WM	15.9 ± 5.45		
	BJ002	10.3 + 0,01					32.8 ± 3.53		
	BJ000						WM		
Spring VIII	**BJN**								
	BJ001						31.0 ± 5.84		
	BJ002								
					6.7 ± 1.30				

Rev/mL- Revertants per milliliter of water ± S.E.M. using Salmonel software; Sampling sites BJN, BJ001, BJ002 and BJ000 - WM – Without Model; Negative Control rev/plate: 28.05 ± 9.46 (TA98-S9), 34.2 ± 6.34 (TA98 + S9); 95.43 ± 26.45 (TA97 - S9), 133.76 ± 22.53 (TA97 + S9); 201.6 ± 74.75 (TA100 - S9), 198.0 ± 57.52 (TA100 + S9); 16.96 ± 7.47 (TA1535 - S9), 17.72 ± 7.12 (TA1535 + S9); N, negative response; Positive Control rev/plate: 4NQO (0.5mg/plate) 157.8 ± 46.94 (TA98 - S9), 692.0 ± 170.25 (TA97 - S9); AZS (5mg/plate) 2131.1 ± 1018.60 (TA100 - S9), 1210.3 ± 619.03 (TA1535 - S9); 2AF (10mg/plate) 1266.9 ± 539.34 (TA98 + S9), 663.0 ± 61.37 (TA97 + S9), 1729.3 ± 816.41 (100 + S9), 1280.1 ± 395,12 (TA1535 + S9).

Table 2: Mutagenic response of water (revertants /mL) in the presence or absence of S9 mix, from Bom Jardim Stream in different seasons

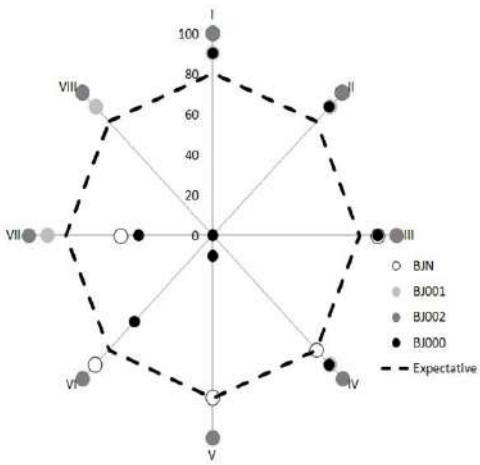

Figure 3: Percentage of survival of *Daphnia magna* exposed to the raw sediment samples.

(Figure 4) shows the total number of neonates per point sampled, over time (circular markers) and the trend of responses for this period (dashed lines), where the low reproductive yield is shown at BJ000. The minimum expected neonate formation in a healthy environment is one thousand neonates. It is observed that at the first sampling time (Dec/98) BJN and BJ002 presented the expected number of neonates, and that later the number of neonates dropped. All the sites sampled during this period indicate a drop in quality as time goes on.

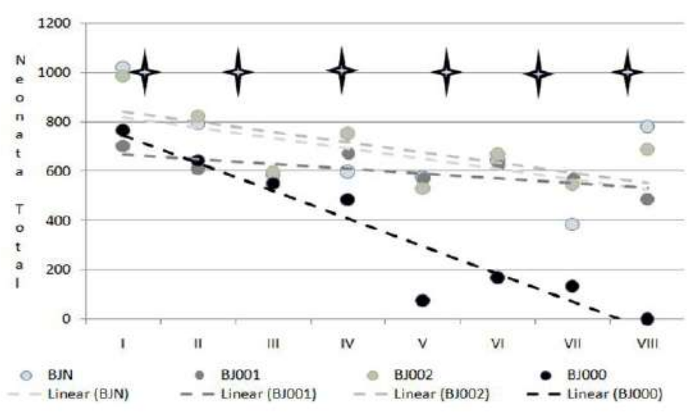

Figure 4: *Daphnia magna* neonates and lines of trends per site over time. The starred line indicates the number of neonates expected in a healthy environment.

Analysis of organic compounds in fish tissue and sediments

Organochlorinated residues were detected in one of the fish samples at BJ000, in which Lindane (0.5 ppt/g), Heptachlor (2.5 ppt/g), Endrin (0.25 ppt/g) and ß-BHC (0.5 ppt/g) were found. Negative responses were observed for the presence of organochlorinated pesticides (Aldrin, α-BHC, ß-BHC, ▲-BHC, DDD, DDE, DDT, Dieldrin, Endosulfan I, Endosulfan II, Endosulfan Sulfate, Endrin, Endrin aldehyde Heptachlor, Heptachlor epoxide, Lindane). The results of the analyses performed in sediment showed the presence of benzene at concentrations close to 1 ppm at BJ002, in two out of five sampling periods (third and fifth sampling). No organic solvents were detected in the other samples.

Embryo-Test in sediment

The results of the exposure of *Danio rerio* embryos in the sediment of area BJ000 of Bom Jardim Stream showed several deleterious effects during their development, compared to the negative control. The data are shown in (Figure 5) where (A) presents the percentage of survival of the embryos exposed at different concentrations of sediment, at different times during their development, and it is possible to verify the high mortality compared to the controls. Embryo sensitivity to sediment toxicity was more significant after 48 hours at all sample concentrations. Figure 5B presents the most relevant percentage of malformations found in the embryos, enabling the identification of a direct relationship between the level of sediment concentration and the variety of malformations, which are most constant as regards Edema (Ed) and Lordosis (Lo). Lethal and sub lethal effects recorded after 96 h of exposure, major effects in relation to exposure concentrations to sediment samples from Bom Jardim Stream point D (Edema tail/ unresorbed yolk sac, Lack of pigments, Helicoidal tail, Deformation of tail and head, Lordosis, eyes Defect. Calculated as means from three independent replicates.

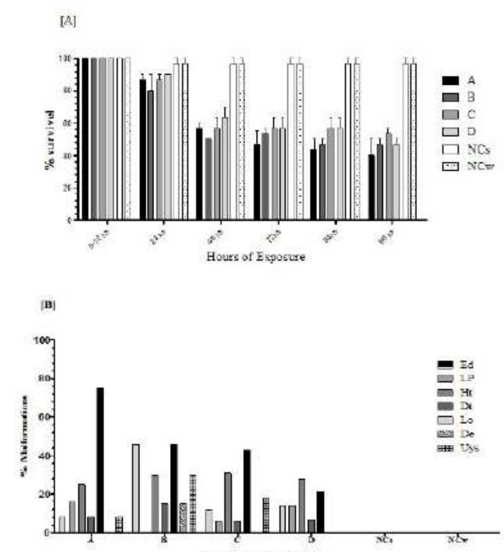

Figure 5: Results of Embryo-Test of native sediment: [A] Percentage of survivors with zebrafish (*Danio rerio*). Survival was analyzed at different times in embryo development. (NCs=Negative Controls –artificial sediment; NCw= Negative Controls - artificial waters). [B] Percentages of malformations: Ed- Edema tail; Uys-unresorbed yolk sac; LP- Lack pigments; lack of pigments Ht- Helicoidal tail; Dt – Deformation of tail and head; Lo- Lordosis; De- Defect eyes.

Discussion

In the surface water of Bom Jardim Stream the different assays showed the marked influence of the petrochemical industry wastes. Cytotoxicity was present in most of the samples analyzed, and was positive in at least one of the assay conditions, in 60% of the BJN samples and 100% of the other sites. The responses were more frequent in the assays in the presence of metabolization, distributed similarly throughout the seasons of the year. During the same period of study, the analyses of the organic extracts obtained from sediment samples showed seasonal variation, and there were a higher percentage of cytotoxic responses in the summer samplings, although there was also a greater presence of the direct action compounds. The high frequency of cytotoxic compounds may have compromised the detection of the mutagenic response, agreeing with the study by Horn et al. [38]. When less than 60% of the bacterial population survives against the tested sample, the visualization of the rare mutagenic event is impaired, which may generate false negative results. The cytotoxic effect of the samples could lead to decreased mutagenic activity in the Salmonella/microsome assay [25].

As to the mutagenic activity of the surface waters, there was a gradient in the percentage of positive responses in the direct assays between 40% and 100%, from the stream source, point BJN, to the mouth, point BJ000. In the tests to evaluate the metabolites, there was a clear impairment of site BJ000 (100% of the positive samples). More frequent responses could be observed in the summer samplings showing frameshift activity and in winter for the base pair substitution mutation. Considering the response as positive/indicative of positivity, when present in at least one assay of the set analyzed, it was possible to observe for direct assays or those with S9 a gradient of 40% to 80% in the percentage of positive responses from the source to the stream mouth (BJ000), but considering the activity positive when one of the assays presented a positive response, the percentage was 100%, and this point was the highest compared to the other sites studied. The values in a mutagenesis index were often close to the negative control of the assay. However, they presented a dose-response curve with significant positivity, predictively of mutagenicity and reproducibility of these responses during the different seasons of the year. These responses were more frequent in summer for frameshift and in winter for base pair substitution mutations.

The presence of benzene detected in sediment samples at concentrations close to 1 ppm at site BJ001, in front of the area where treated effluent is disposed of on the soil, marking the influence in the stream should also be emphasized. Comparatively, a gradient of mutagenic activity, although less intense, varying from 20% to 60% from BJ000 to the stream mouth, can also be observed [29]. Evaluating the sample results in revertants/mL it was possible to observe: for BJN an order of magnitude from 2.9 to 159.5; for BJ002 from 4.1 to 64.9; at BJ001 from 2.7 to 27.2 and for BJ000 from 1.9 to 109.5. Considering the classification made by [63], utilizing the number of revertants/L equivalents of the sample as parameter (rev/L), the values observed would be from moderate (values between 500-2500 rev/L) to high (values up to 5000 rev/L) mutagenicity, in the minimum values observed per sampling site and extreme mutagenicity (above 5000) in the highest values. [16] Used the same classification to compare studies on the mutagenic investigation of water sources on different continents. In this review [16], agreed with the studies performed by [25] in Brazil concerning the Cai River area, including the mouth of Bom Jardim Stream during the period of November 1993-May 1995, where BJ000 was classified with extreme mutagenesis values.

In the study correlating the physicochemical parameters with the biological ones it was found that: (a) there may be an elevation of sample cytotoxicity in assays in the presence of metabolization for lower pH values; (b) this biological effect also arises, appears in the presence of higher turbidity values (assays with the S9 fraction) and zinc (Zn) (total of events); (c) as to mutagenesis, there was an elevation in the total number of events with lower pH values. Except for Zn, it could be observed that the general parameters of the sample with pH and turbidity presented a correlation, indicating that the events detected by the biological assays depend on other chemical variables.

The study shows that Bom Jardim Stream presents ecotoxicological degradation as the first body of water to receive the impact of the petrochemical industry area that is being analyzed, and that was also found in previous studies [52]. The responses observed for mutagenic and cytological activity in water, as well as those described in [29] for sediments, show the validity of the Salmonella/microsome assay to diagnose degradation of the environmental quality of this stream. The studies already developed covering the main river in the basin, Caí River [22,49,52], showed assay sensitivity as a major tool for environmental diagnosis in areas under anthropogenic impact from petrochemicals.

The Petrochemical Complex, is also under the influence of airborne pollutants, as demonstrated by [64-67] and the organic compounds adsorbed to air particles induced DNA adducts; the carcinogenic Polycyclic Aromatic Hydrocarbons (carc-PAHs), were mostly responsible for the genotoxic activity, contributing to 45%–50% of all DNA adducts induced by these complex mixtures. In [25] the genotoxicity of Caí River water sampled during two periods was analyzed using the *Salmonella*/microsome assay. The percentage of mutagenic activity was higher at the sites sampled in front of the Petrochemical Complex in both periods, but there were more significant mutagenic responses in the first assessment and in the cytotoxic samples during the second period. At site BJ000, which presented a percentage of 42% of mutagenic samples in the first study, only 10% were mutagenic during this second phase of the study. Cytotoxic activity, however, was raised from 10 to 70% of the samples evaluated in the current monitoring. The mutagenic activity values measured in rev/mL could only be measured by sampling in TA97a assays in the presence of S9, with values of 18 rev/mL, considered moderate for mutagenicity [16] This ratio of increased percentage of cytotoxic samples (from 22% to 63%) to decreased samples with mutagenic activity (from 34% to 18%) is observed in this study for other sites assessed in Caí River.

Previous studies showed that *Daphnia magna* is an excellent indicator organism to evaluate sediment toxicity [51,28]. The number of neonates generated from the samples collected indicates a drop in the number of individuals generated between the first and the last sampling, indicating that the capacity to maintain the fauna in the area may diminish. The data shows a worse quality at point BJ00 located downstream from the other points, with the presence of chronic toxicity at all sites sampled. While points BJN and BJ001 present the best yields, BJ000 generated fewer individuals in all samplings. This response was expected, since all points' upstream influence Point BJ000. The trend lines traced for the reproduction event shows a progressive drop in the quality of the environment in the area studied.

The *Danio rerio* fish has been a tool to analyze the quality of aquatic environments and in this study it showed promising results regarding sensibility to the different toxic compounds found in the sediment, corroborating other studies where zebrafish embryos were used as toxicity indicators [1,46,47,68,69]. The *Danio rerio* embryos exposed in the sediment sample from Bom Jardim Stream showed high mortality and morphological and physiological alterations at different phases of their development, and the most evident ones were scoliosis and lordosis. Changes were found in the heartbeat and also in development, which consequently altered the normal embryo hatching process as related to the negative control. [70], in his work, showed that there is a correlation between toxicological data and different levels of water and sediment contamination, which was shown in our work where the higher concentrations of exposure had a greater number of deformed individuals [71] in his work, identified biological effects after exposure to environmental water samples. Zebra fish embryos were exposed to water samples of five different sites. On cluded that, the effects of the environmental water samples, were caused by metal mixture toxicity rather than by other unknown toxicants. The embryos were sensitive enough to develop malformations and/or show altered swimming activity in several exposure groups [72,73].

This work takes another look at the importance of evaluating areas around petrochemical industry activity, since the compounds originating from these activities may directly affect the water sources, and are a threat to the aquatic ecosystem, and therefore environmental monitoring intermingling physicochemical data with biological data is essential to evaluate the toxic effects on a living organism [74,75].

Conclusion

The present retrospective study showed evidence of the impairment of Bom Jardim Stream as the first area of the Caí River basin to suffer the impact of the treated liquid effluents of a Petrochemical Industrial Complex [76]. The mutagenic and cytotoxic activities detected in the surface water and sediments showed evidence of the sensibility of the *Salmonella*/microsome assays, as well as the survival and reproduction responses for *D. magna*. These effects, both mutagenic and toxics can be attributed to the presence of several metals and organic compounds originating in the petrochemical industry and their complexes, or other environmental factors. Genotoxins in industrial wastes may possibly be transferred into the trophic chain and the effect is biomagnifications contaminating the water resources and soil systems. In this way, the assays using zebrafish showed sensitivity to detect sediment contamination in the area under the greatest industrial influence, suggesting that it is adequate as a model to evaluate impacted areas. Since the diagnosis was performed in an area of historical discharges (1995-2005) [77], the applicability observed for the assays, associated with the widely disseminated use on different continents and the standardization of procedures at a worldwide level qualify them as parameters of choice for inclusion in environmental legislation to control environmental quality in areas that suffer anthropogenic impact.

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Compliance with Ethical Standards

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Ethical standards

We confirm that the study performed was strictly in accordance with acceptable ethical procedures. Ethical approval: All applicable international, national and institutional guidelines for the care and use of animals were followed.

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