Water pollution monitoring of the Lujan River (Argentina): chemical analyses and hepatic biomarkers in *Lithobates catesbeianus* tadpoles

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Abstract: The toxicological water quality of the Lujan River (Argentina) was monitored during one year seasonal samplings. Water samples were collected at two points and their toxicological profiles were compared: S1 (reference site), located downstream of a major city and S2, located further downstream, beyond a joint urban sewage and industrial discharge point. A number of abiotic parameters were determined and three water quality indices (WQIs) calculated on the samples. Laboratory toxicity bioassays were conducted exposing *Lithobates catesbeianus* larvae to samples; a third group of animals were exposed to tap water (controls). Hepatic biomarkers were determined: catalase (CAT), glutathione-S-transferase (GST) and superoxide dismutase (SOD) activities, lipid peroxidation (LPO) and GSH content; CF and HSI were calculated. The scores of the WQIs corresponded to a high pollution condition at both sampling points. ANOVA showed significant differences between sampling sites and controls mainly in CAT activity, GSH content and GST in autumn.

Keywords: peri-urban river pollution; Lujan River (Argentina); *Lithobates catesbeianus* tadpoles; hepatic biomarkers; oxidative stress.

Reference to this paper should be made as follows: Ossana, N.A., Castañé, P.M., Eissa, B.L. and Salibián, A. (2017) 'Water pollution monitoring of the Lujan River (Argentina): chemical analyses and hepatic biomarkers in *Lithobates catesbeianus* tadpoles', *Int. J. Environment and Health*, Vol. 8, No. 2, pp.150–163.

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1 Introduction

Peri-urban water bodies' qualities are at risk owing to the constant discharge of a complex mixture of substances generated as a result of anthropogenic activities. Therefore pollution becomes stressful for the biota, and may involve risks for humans (Lushchak, 2011; van der Oost et al., 2003).

The evaluation of the toxicological quality of a water body using physicochemical analyses has been shown to be insufficient and should be supplemented with information provided by measurements of biochemical parameters taken simultaneously on sentinel species (Sánchez and Porcher, 2009); they exhibit a number of enzymatic and non-enzymatic protective mechanisms to counteract the adverse impacts of the assayed samples causing alterations to critical parameters beyond their steady-state equilibria. Thus biochemical biomarkers can be used to evaluate the consequences involved in the interaction between a biological system and the physicochemical profile of a particular environment (Conti, 2008).

A variety of parameters have been used as sensitive biomarkers in particular for oxidative stress evaluation in aquatic species (Cazenave et al., 2009; Maggioni et al., 2012). Biochemical and morphological parameters are increasingly being used as 'early warning' (sub-lethal) signs to assess the environmental stress conditions (Lushchak, 2011; Schlenk et al., 2008). The study of aquatic pollution by integrating environmental chemical information for water and test organism biomarkers is a methodology that was shown to be adequate for the study of stressed environments.

The objective of this study has been to evaluate the usefulness of integrating water physicochemical profile of the samples with morphological parameters and hepatic

biomarkers of *Lithobates catesbeianus* pre-metamorphic tadpoles to assess comparatively the toxicological quality of surface water in a particular area of the Lujan River, before and after a complex urban-industrial effluent discharge point with significant signs of chronic anthropogenic environmental stress.

Amphibians are sensitive to a large number of environmental pollutants such as pesticides, heavy metals and polychlorinated biphenyls among others (Dornelles and Oliveira, 2014; Ferrari et al., 1998, 2005; Lajmanovich et al., 2014; Venturino and Pechén de D'Angelo, 2005); these species are considered reliable indicators of environmental quality owing to their biphasic life (aquatic and terrestrial) and semi-permeable skin.

Our particular experience with *L. catesbeianus* showed its value to be used in aquatic toxicity bioassays of peri-urban water bodies as test species (Ossana et al., 2010, 2013; Ossana and Salibián, 2013) standing out for display sensitivity with clear effects after acute exposures.

We have previously carried out studies with the aim of assessing the quality of surface water samples from different peri-urban water courses in the Buenos Aires Metropolitan Area by evaluating the impacts on morphological, behavioural and physiological biomarkers of fish and amphibian larvae (Eissa et al., 2010; Ossana, 2011; Salibián, 2006).

2 Materials and methods

2.1 Animals

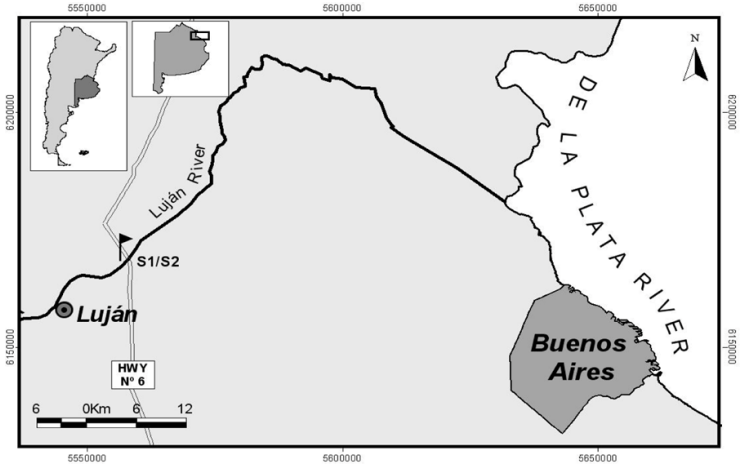
Test organisms were bullfrog (*L. catesbeianus*) pre-metamorphic larvae (body wet weight: 2.5 ± 0.1 g; length: 6.1 ± 0.1 cm; $n = 155$). They were obtained from a commercial supplier and had never been exposed to pollutants. Prior to the assays, tadpoles were acclimated to laboratory conditions for seven days in glass aquaria under a continuous flow through system with tap water (TW) at 21 ± 1°C, permanent aeration, feeding *ad libitum* once a day on commercial fish pellets. According to the season, the photoperiod during the bioassays was adjusted to 16 : 8, 12 : 12 and 8 : 16-h light–dark cycles.

2.2 Description of the study area

The Lujan River is 135 km in length, located in east-central Argentina, close to the City of Buenos Aires, with a catchment area covering 2940 km^2. The river is tributary of the Rio de la Plata; it is associated to high urban-industrial concentration in the middle and lower basin, exhibiting signs of anthropogenic environmental stress.

The study was conducted on samples taken at two sites, S1 and S2 (Figure 1), about 60 m away from each other, 70 km from the headwater (S1: 34°31′15.20″S and 50°02′15.50″W; S2: 34°31′13.49″S and 59°02′12.67″W) and 12 km downstream of the city of Lujan (Buenos Aires Province). A permanent discharge point involving a mixture of domestic (sewage) and industrial (beer factory and paper mill) effluents was located between S1 and S2. S1 was considered the reference site representative of the upstream conditions before the discharge point.

Figure 1 Geographic location of Luján City and sampling sites



2.3 River water sampling. Environmental conditions

Water samplings (15–20 cm depth) were performed in March (summer), June (autumn), August (winter) and December (spring). The mean air temperature in the study area for each month was 20.8, 10.3, 18.5 and 17.8 (°C), respectively. Rainfall rate the week before each sampling was 6, 34, 1.5, and 96 mm, respectively; no rain was reported on sampling days.

Water samples (three samples of 500 ml of each site) for physicochemical analyses were collected in clean polyethylene containers and stored at 4–8°C until analysed (3–5 days). Aliquots for heavy metal determinations were taken and kept acidified with HNO_3 ($pH \leq 2$), whereas those for pesticide determinations were collected in amber-colour glass bottles.

A sample of 100 L for bioassays was simultaneously collected at each site in polyethylene containers and stored at 4–8°C.

2.4 Toxicity bioassays

Two groups of 10–18 tadpoles were exposed to the river water samples (S1, S2) for 6 days; a third group was exposed to tap water (TW). All aquaria (24 L) were aerated and a constant flow was maintained. During the assays, the aquaria water medium was renewed every 48 h. Two hours prior to renewal, animals were fed an amount equivalent to 2% bw; remaining food was then removed.

2.5 Physicochemical analysis of the river water samples

2.5.1 Field determinations

Temperature, pH and conductivity were measured using a Hanna portable sensor and an Orion EA pH meter.

2.5.2 Laboratory determinations

Alkalinity and chloride concentration were quantified by titrimetry (H_2SO_4 and $AgNO_3$). Ammonium (NH_4^+), nitrites (NO_2^-), nitrates (NO_3^-), soluble reactive phosphorous concentrations and chemical oxygen demand (COD) were determined using colorimetric methods. Dissolved oxygen (DO) was estimated using the Winkler method and 5-day biochemical oxygen demand (BOD_5) as the difference between initial and fifth day oxygen concentration. All analyses were performed in duplicate following the standard methods (APHA-AWWA-WEF, 2005).

2.6 Water quality indices

The WQI-B (Berón, 1984) is an indicator of domestic pollution and WQI-I (Lacoste and Collasius, 1995) refers to industrial pollutants. The WQI-PW (Pesce and Wunderlin, 2000) is based on a larger number of parameters.

2.7 Pesticides

Pesticide screening was conducted on three samplings by high resolution capillary gas chromatography (Hewlett Packard; 61530 Plus A6890) equipped with appropriate capture detectors (ECD, FPD and NPD). Screening included the following pesticides: *Organochlorines*: Aldrin, α-, β- and γ-Chlordane, DDT and metabolites, Dieldrin, α- and β-Endosulfan, Endrin, Heptachlor, Heptachlor epoxide, Hexachlorobenzene, α- and β-Hexachlorocyclohexane, Methoxychlor and Mirex; *Organophosphates*: Bromophos, Chlorfenviphos, Chlorpyriphos, Coumaphos, Diazinon, Ethylbromophos, Ethion, Fentrothion, Malathion and Methylparathion. Detection limits ($\mu g\ L^{-1}$) were 0.03 for organochlorines and 0.02 for organophosphates.

2.7.1 Heavy metals

Manganese (Mn), Zinc (Zn), Copper (Cu), Chromium (Cr), Lead (Pb) and Cadmium (Cd) concentrations in river and TW samples were determined by atomic absorption spectrophotometry (Shimadzu 6700). Detection limits ($\mu g\ L^{-1}$) were as follows: Mn (1.0), Zn (10), Cu (1.0), Cr (0.5), Pb (1.0) and Cd (0.5). Results were expressed as the mean values of two readings.

2.8 Biological and biochemical determinations

2.8.1 Morphological parameters

Following the exposure period, tadpoles were anaesthetised in ice-water, weighed (g) and measured (cm) to calculate the Condition Factor [$CF = (100 \text{ body weight} \times \text{total length}^{-3}$]. The liver of each animal was removed and weighed (g) to calculate the Hepatosomatic Index [$HSI = 100 \text{ liver weight} \times \text{body weight}^{-1}$].

2.9 Biochemical parameters

2.9.1 Antioxidant enzyme activities

Supernatants from hepatic tissue homogenates were prepared following Ossana et al. (2010); the post-mitochondrial fraction (PMF) of the homogenate was used to measure catalase (CAT), glutathione-*S*-transferase (GST) and superoxide dismutase (SOD) activities and glutathione (GSH) content.

CAT (EC 1.11.1.6) was measured following the method proposed by Baudhuin et al. (1964), evaluating H_2O_2 consumption over time. Change in absorbance at 25°C was recorded at 240 nm for 60 s.

GST (EC 2.5.1.18) activity was determined using the Habig et al. (1974) method, using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Change in absorbance at 25°C was recorded at 340 nm for 2 min.

SOD (EC 1.15.1.1) was measured as suggested following the McCord and Fridovich method (1969). Activity was reported as mixture ability to inhibit 50% of cytochrome C reduction by competition with SOD for the superoxide anion radical formed by the xanthine/xanthine oxidase system. Change in absorbance at 25°C was recorded at 550 nm.

2.9.2 Biotransformation biomarkers

GSH content was determined using the Ellman (1959) method. PMF was precipitated with TCA (10%) and centrifuged at $10{,}000 \times g$ for 10 min at 4°C. The supernatant was used for GSH measurement as acid-soluble thiols (AST), using 5,5′-dithiobis-(2-dinitrobenzoic acid); absorbance was measured at 412 nm. AST were quantified using a calibration curve with pure GSH as standard.

2.9.3 Lipid peroxidation (LPO)

LPO was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) under acidity and heat conditions according to Oakes and van der Kraak (2003). The chromogen formed was measured by fluorometry (Shimadzu RF-540 Fluorometer) by excitation at 515 nm. Lipid peroxide concentration was calculated using tetramethoxypropane as an external standard.

Liver protein content was determined following the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

The spectrophotometric measurements were carried out in a Metrolab 1700 UV-Vis unit.

Reagents were obtained from Sigma-Aldrich; solutions were prepared using Milli-Q water.

All measurements were carried out in triplicate and calculations made on the basis of the average percentage normalised values. Enzyme activities were calculated in terms of sample protein content.

2.10 Statistical analyses

Biomarkers data were reported as a percentage (mean ± SEM) relative to controls. Normality and homoskedasticity of the variance were checked by the Kolmogorov–

Smirnov and Bartlett tests, respectively. Statistical differences between groups (controls-S1-S2 for each sample) were determined using one-way analysis of variance (ANOVA) followed by a Multiple Comparison test (Tukey); Kruskal–Wallis testing was used when data did not meet the required conditions for ANOVA (Zar, 2010). Significance level was $p < 0.05$.

Analyses were performed using the STATISTICA version 6.0 software package.

3 Results

3.1 Chemical profile of river samples

The results of the physicochemical analyses of the river samples are shown in Table 1; some of them revealed considerable differences between the two sampling sites. pH was fairly stable, albeit slightly alkaline, whereas conductivity, hardness and total alkalinity were always higher than TW controls.

DO levels were very low in all river samples. COD:BOD_5 ratio was indicative of a significant contribution of non-biodegradable organic matter. Other indicators of domestic, municipal and industrial discharges (phosphorus, NH_4^+, NO_2^-) were almost always higher than TW controls and maximum permissible quantities established in the Argentine legislation for protection of freshwater life. Heavy metals showed high variability without a uniform pattern of changes, although in several cases the levels were above those allowed under local legislation for the protection of aquatic life.

Pesticide concentrations data were in all samples below the detection limit of the analytical techniques; therefore were not included in Table 1.

The three WQIs of river samples ranged between 3 and 6 (indicative of slight to moderate pollution) without important differences between sampling sites and season; TW indices were between 8 and 9.

3.2 Bioassays

3.2.1 Morphometric and biochemical parameters

Results are shown in Figure 2. No tadpole mortality was recorded at the end of the exposure period. CF did not exhibit any significant variation; HSI remained stable in most cases increasing only in S2-spring as compared to controls.

Animals presented alterations in liver protein content, enzyme activities and biotransformation biomarkers.

Liver proteins showed changes only in tadpoles exposed to S1 in autumn and to S2 in winter. CAT and GST activities increased significantly in autumn for both sites as compared to controls. GSH content showed a significant increase in summer and autumn mainly in S2 samples while in winter and spring this content decreased slightly. TBARS increased in all samples compared to controls except spring, in S1 for summer and autumn and in S2 for winter. SOD activities remained close to control values was slightly high relative to controls.

Table 1 Physicochemical parameters and water quality indices of Lujan River and tap water (TW)

Parameter	*Units*	*Summer*		*Autumn*		*Winter*		*Spring*		*TW*	*Argentine guidelines**
		S1	*S2*	*S1*	*S2*	*S1*	*S2*	*S1*	*S2*		
pH		7.8	7.9	8.5	8.6	8.3	8.0	7.7	8.0	8.1–8.5	6.5–10
Conductivity	$\mu S.cm^{-1}$	1090	1500	1850	1667	2350	2300	913	1570	920–1000	
Hardness	mg $CaCO_3.L^{-1}$	130	120	170	150	190	150	120	90	60–95	
Total Alkalinity	mg $CaCO_3.L^{-1}$	484	499	600	772	655	946	437	946	380–450	
Chlorides	mg $Cl^-.L^{-1}$	160	190	205	135	238	167	62	35	13–31	
Dissolved oxygen	mg $O_2.L^{-1}$	1.5	1.7	3.3	5.3	1.6	0.8	2.8	0	6–11	
Ammonium	mg $N–NH_4^+.L^{-1}$	5.8	5.8	4.6	3.1	4.3	2.4	2.3	1.9	0.4–1.6	0.05–0.47
Nitrites	mg $N–NO_2^-.L^{-1}$	0.8	0.8	0.2	0.2	0.8	0.5	0.5	0.7	0.02–0.1	0.06
Nitrates	mg $N–NO_3^-.L^{-1}$	0.7	0.7	1.5	3.7	0.8	2.8	0.3	0.1	3.6–3.8	
Phosphorus	mg $P–PO_4^{3-}.L^{-1}$	0.6	1.1	1.1	0.7	1.3	1.3	1.2	2.7	0.1–0.3	<1.0 #
Biochemical oxygen demand (BOD_5)	mg $O_2.L^{-1}$	2.1	1.8	6.9	8.4	5.6	10.3	14.5	3.6	0.7–1.2	<50
Chemical oxygen demand (COD)	mg $O_2.L^{-1}$	33	81	59	67	65	96	36	>150	0	<250
COD/BOD_5		15.8	45.8	8.5	8.0	11.5	9.3	2.5	>41.7	0	

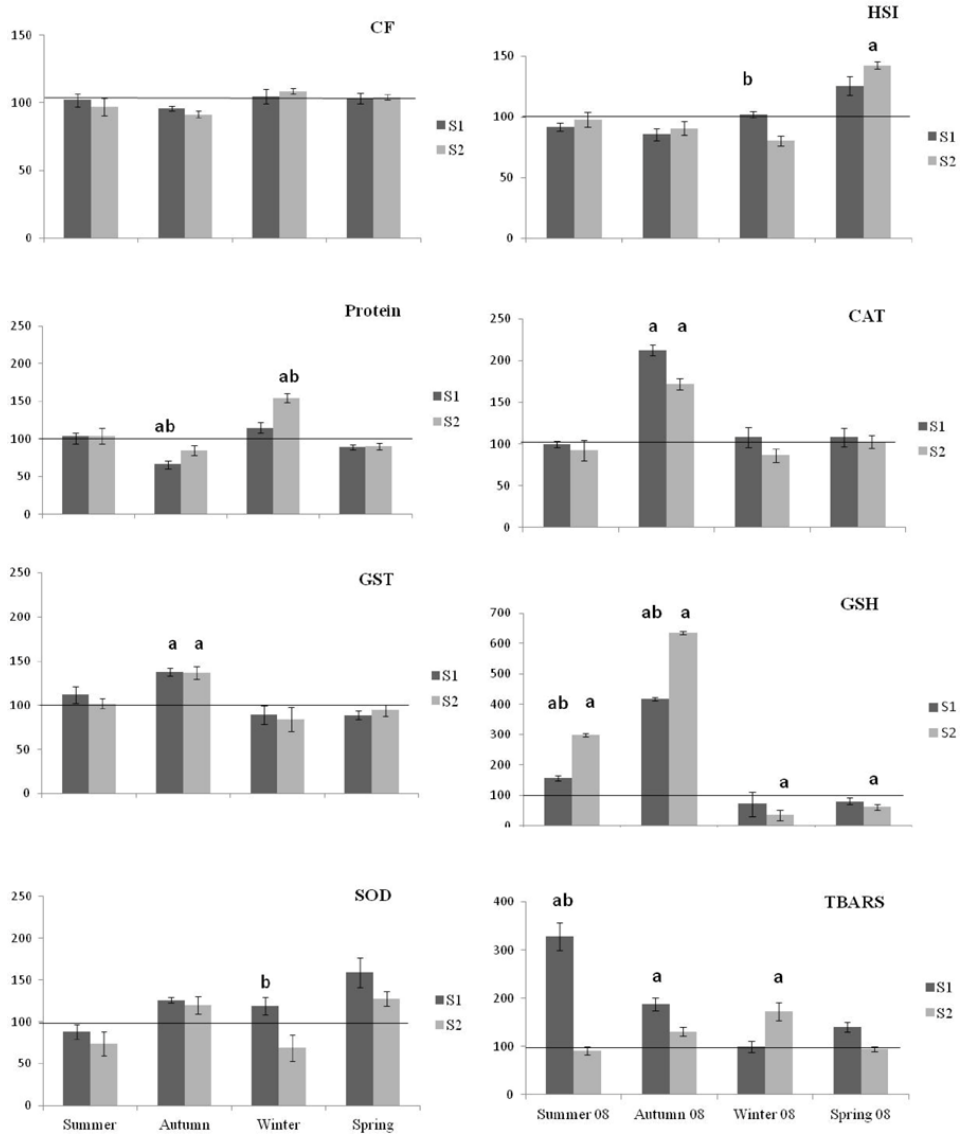
Table 1 Physicochemical parameters and water quality indices of Lujan River and tap water (TW) (continued)

		Summer		*Autumn*		*Winter*		*Spring*		*TW*	*Argentine guidelines**
Parameter	*Units*	*S1*	*S2*	*S1*	*S2*	*S1*	*S2*	*S1*	*S2*		
Heavy metals:	$\mu g.L^{-1}$										
Mn		101	96	20	<5	31	13	44	<5	ND	100
Zn		88	35	26	15	26	17	65	18	33	30
Cu		22	D	<5	<5	3	4	180	32	D	2–4
Cr		D	D	11	6	5	3	3	3	D	2.5
Pb		41	D	<5	<5	<2	<2	<2	<2	23	1
Cd		D	D	<1	<1	<1	<1	<1	<1	<1	0.1–0.4
Water quality indices:											
WQI-P[a]		4.6	5	4	4	5	3	3	5	9	
WQI-I[b]		6.5	5	6	6	6	6	6	4	9	
WQI-PW[c]		5	5	5	4	4	4	4	3	8	

[#]Total phosphorus.

Results are expressed as means of two readings. D: detected; ND: no detected; TW data as range. [a]Berón (1984); [b]Lacoste and Collasius (1995); [c]Pesce and Wunderlin (2000). *Surface water Argentine Guidelines for protection of aquatic life.

Figure 2 Physiological and liver biochemical biomarkers of *L.catesbeianus* tadpoles exposed for six days in surface water samples taken from the Lujan River (S1–S2)



Values are expressed as a percentage relative to control; (n = 10–18). The solid line represents the controls. Data (mean ± SEM) with triplicate measurements are shown. [a]mean significant difference from control. [b]means significant difference between sampling sites ($p < 0.05$).

4 Discussion

Since the chemical evaluation for toxicological assessment of complex mixtures like river water samples could be insufficient for their characterisation, the most appropriate way to achieve this purpose will require an integrated, physicochemical and biological approach

in addition to a monitoring program to evaluate water quality evolution over time. Toxicity evaluation based on bioassays may supply more realistic information. In our study assays both on control and exposed tadpoles to river water were carried out simultaneously, thus to avoid the influence of known seasonal variability in the biomarkers.

Several studies carried out within that framework (Sánchez Caro, 2010) have described some of the physical, chemical and microbiological features of the watershed, the temporal variation involved and the impacts of the industrial effluents discharged into the main course of the river. These studies have also followed water quality evolution on the basis of different indices and estimated the waters' self-purification capacity (Giorgi, 2001). More recently Ossana and Salibián (2013) reported a significant increase in the micronucleus frequency in peripheral erythrocytes of *L. catesbeianus* tadpoles after their exposure to Lujan River samples.

The results obtained in this study confirm our previous data in connection with this and other species of amphibians, as well as those by other authors, who have demonstrated their usefulness of these aquatic life-forms as sentinel organisms in ecotoxicity studies (see Ferrari et al., 1998, 2005; Venturino et al., 2003). In our case, we have supplied additional evidence to show that the test species used and the protocol followed may be suitable to provide a more precise description of the toxicological quality of environmental water samples.

The sampling sites were located beyond the point where the river flows through the city of Lujan, an urban conglomerate of approximately 107,000 inhabitants. When compared with that of TW controls, the physicochemical profile of the river water samples showed an important deterioration in quality. DO levels indicated that the river water could be characterised as permanent hypoxic condition, which is ecologically serious for aquatic species whose critical oxygen requirement level for development is close to 4 mg L^{-1}. Furthermore, hypoxia conditions, in addition to high concentration of nitrogen compounds and heavy metal levels, are known to be environmentally toxic to aquatic fauna. With regard to the condition of stable hypoxia detected in the analysed samples, it is interesting the evidence recently reported by Jenny et al. (2016); these authors concluded that the hypoxic condition of aquatic ecosystems might be a consequence of the increased human activity and the discharge of nutrients, without correlations with changes in environmental changes of temperature.

The comparison between BOD_5 values and COD values highlight the fact that, in all cases, the river was in S1 oxygen-deficient providing insufficient oxygen supply to the biota.

In all cases, pH was slightly alkaline, which matches the considerably high nutrient concentrations. Conductivity was relatively high while nitrogen compound and phosphate concentrations were in almost all cases higher than the maximum quantities allowed under local legislation. It is also important to point out that the analyses conducted on the samples revealed the presence of certain heavy metals which are known to be toxic to aquatic life, at much higher concentrations than those allowed under the local legislation.

As for the WQIs calculated for both sampling sites, the values were around 3–6 indicating high pollution for both sites. Wachs (1998) proposed a water quality classification system of rivers according to their concentrations of several heavy metals. In this case, after considering the measured concentrations of heavy metals that were above the regulatory guidance levels (Table 1), the toxicological quality of the river samples corresponded to grades III and IV (very heavily polluted). Thus, the WQIs and

the analysed liver biomarkers of the test organism were indicative of an important deterioration of the surface water quality of the Lujan River.

The results of the analysis of pesticide concentrations in the samples suggest that the changes recorded for certain biomarkers cannot be attributed to the presence of such products.

The morphological parameters of the larvae were not sensitive biomarkers of the ecotoxicity condition of the samples. CF is a non-lethal index providing an indication of the animals' health, nutritional status and ability to tolerate the effect of environmental stressors (Mayer et al., 1992). Our results showed that the CF of *L. catesbeianus* did not exhibit significant changes after 6 days' exposure to the river samples. Although HSI is accepted as a useful simple toxicity biomarker, in this case it remained stable in most cases.

Liver response is considered representative of the metabolic impact of the toxics present in the media. Our study included the measurement of a set of biomarkers used to assess the balance between enzymatic and non-enzymatic antioxidant defence systems and the production of ROS in the liver. GSH content and lipid peroxidation processes were determined. It was also found that in some instances there were cases of TBARS induction, and this was higher in tadpoles exposed to environmental samples, suggesting cell damage effects after a relatively short-exposure time.

Most of the samples exhibited only discrete GST activity enhancement; this result could be explained by the fact that the levels of pesticides, which are considered GST substrates were negligible. However, there were differences in particular during the autumn, when TBARS, GSH and CAT were high both in S1 and S2. It is worth mentioning that the recorded environmental temperature in autumn was very low, thus affecting the availability of some toxics in the environment. We conclude that antioxidant enzyme activity may provide effective protection against the adverse effects of oxidative cellular damage due to the complex compositions of the effluents poured in the river. A similar response to that encountered for GST was recorded for CAT while SOD activity was close to control response in almost all cases.

Our results indicate that the effluents released in S2 had also mainly sub-lethal effects on the hepatic biomarkers.

Acknowledgements

The work was supported by the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires and Universidad Nacional de Luján, Argentina. We thank Mr. Sebastián Martínez Zabala for generously providing the larvae used in the bioassays.

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