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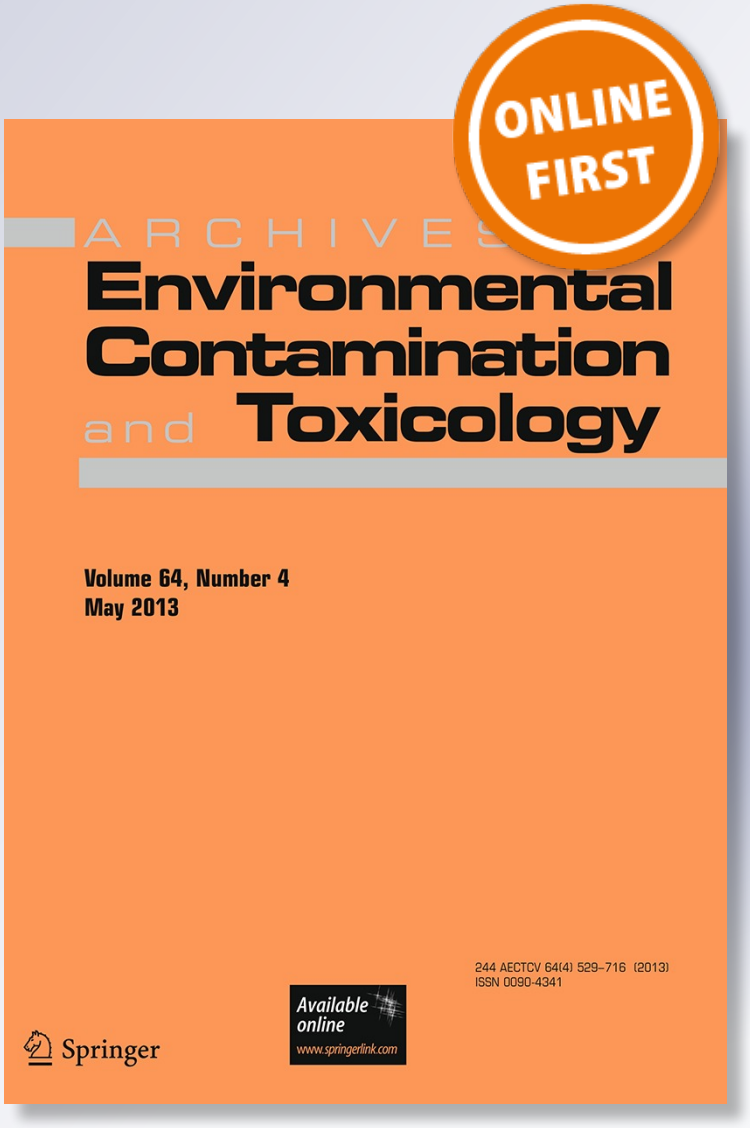


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# Use of *Lithobates catesbeianus* Tadpoles in a Multiple Biomarker Approach for the Assessment of Water Quality of the Reconquista River (Argentina)

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**Abstract** The water quality of the Reconquista River (Argentina) water was monitored between 2009 and 2010 by means of a multiparametric approach. This periurban river is affected by agricultural, urban, and industrial discharges. Water samples were collected at a dam located in the headwaters and at 18 km downstream (M). Physicochemical profile and two water-quality indices (WQIs) were determined. Laboratory bioassays were performed by exposing *Lithobates catesbeianus* tadpoles to environmental samples for 96 h and determining the following parameters: (1) brain: acetylcholinesterase (AChE) activity; (2) gill: catalase and glutathione-S-transferase (GST) activities and glutathione (GSH) content; (3) liver: CAT and GST activities, superoxide dismutase, lipid peroxidation, and GSH content; (4) condition factor and hepatosomatic index; and (5) genotoxicity [micronucleus (MN) test in erythrocytes]. Physicochemical profile and WQIs corresponded with extensive pollution in M. Important temporal and spatial variability in biomarkers of tadpoles exposed to samples was found. Multivariate analyses showed that AChE in brain, MN frequency, liver and gill GST activities, and GSH content were key biomarkers.

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Periurban rivers are examples of ecosystems conditioned by the anthropogenic activity taking place on their margins and their areas of influence. A combination of physical, chemical, and microbiological indicators have been traditionally used for the assessment of quality. These pollution indices, also expressed as water-quality indices (WQIs), are useful for warning about health and environmental risks and for the determination of maximum allowed concentrations. However, the information acquired is not complete because biotic and abiotic interactions are not considered. Bioassays allow physicochemical information to be complemented with biological information to determine the consequences of exposing an organism to a polluted environment. A biomarker is defined as any biological response of a test organism after its exposure to an environmental sample. Thus, biochemical, physiological, histological, morphological, and behavioural measurements (Eissa et al. 2009) are considered biomarkers. Biomarkers can be used to measure the interaction between a biological system and physicochemical environmental agents (van der Oost et al. 2003; Walker et al. 2006; Conti 2008) and as sensitive tools to assess the adverse effects of pollutants on natural populations or communities. The use of biomarkers for monitoring environmental quality has gained considerable interest in many cases (Chèvre et al. 2003a; Cazenave et al. 2009; Falfushynska et al. 2010; Trujillo-Jiménez et al. 2011). Lately, efforts of ecotoxicologists have been focused toward the identification of specific and early biomarkers of exposure or effect on sentinel species on plurispecific ensembles by measuring their modifications after being exposed to particular toxics or mixtures of toxics on a variable scale of time and chemical complexity (Lagadic et al. 1997; Monferrán et al. 2011; Bonnineau et al. 2012). Thus, changes in biomarkers parameters as integrated responses of multiple changes

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occurred in test organism become a reliable signal of the degree of deterioration of a given environment.

In natural environments, contaminants are usually present as complex mixtures, and there is no single biomarker that can give a complete diagnosis of environmental degradation. The use of a set of biomarkers may be useful to evaluate the various responses of an organism under the stress of a mixture of pollutants (Maggioni et al. 2012). A variety of systems have been used as potential biomarkers of oxidative stress, which may occur if the balance between oxidants and antioxidants is disrupted either by excessive accumulation of reactive oxygen species (ROS) or by depletion of antioxidant defenses (Valavanidis et al. 2006). Most environmental pollutants (natural or xenobiotic compounds) enhance the generation of ROS, which react first with susceptible biological macromolecules and then induce cytotoxicity (Di Giulio et al. 1995; Repetto Jiménez and Repetto Kuhn 2009). Lipid peroxidation (LPO), DNA damage, enzyme inactivation, and hormone oxidation are indicators of cell oxidative damage and examples of the toxicity mechanisms of contaminant-induced ROS and are involved in pathological processes (Newman and Clements 2008). Cells exhibit enzymatic and nonenzymatic protective mechanisms to counteract factors that cause alterations in their critical parameters beyond their steady-state equilibrium. Some antioxidants—such as tripeptide glutathione (GSH) and enzymes such as glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), and acetylcholinesterase (AChE)—are specific scavengers of ROS (Monserrat et al. 2007; Viarengo et al. 2007; Lushchak 2011).

The present study focused on the Reconquista River. Its drainage area is located in the pampasic region and covers $\sim$1,670 km$^2$. The river begins at the confluence of the Durazno, La Choza, and La Horqueta streams. The water of these streams is held by the Ingeniero Roggero Dam, 5.1-km long, creating a lake of 460 ha. The river begins at the dam and flows for $\sim$82 km before flowing into the Lujan River, which in turn flows into de La Plata River (Borthagaray et al. 2001; Rigacci et al. 2013).

The experimental design used integrated changes in biochemical and genetic parameters of test organisms as well as the water physicochemical profile. The test organisms used in the bioassays were premetamorphic tadpoles of *Lithobates catesbianus* exposed to water samples under laboratory conditions. In addition, we generated a baseline of all biomarkers in tadpoles exposed to tap water (TW). The aim of this work was to evaluate the usefulness of a battery of enzymatic, nonenzymatic, and genetic biomarkers in *Lithobates catesbeianus* tadpoles to assess the environmental condition on the Reconquista River at two different sites, with different pollutants, as well as to point out which biomarkers and tissues are key in order to use them in future monitoring programs of peri-urban aquatic systems.

*Lithobates catesbeianus* has an extensive distribution and is relatively easy to handle and acclimate to laboratory conditions. It is exotic for the local herpetofauna, but its distribution has expanded due to its great colonizing capacity and ability to adapt to any kind of freshwater body. It was introduced to be used in aquaculture ventures, as an ornamental species, and/or for a biological control (Akmentis and Cardozi 2009).

## Materials and Methods

### Animals

Bullfrog (*L. catesbeianus*, syn. *Rana catesbeiana*) premetamorphic larvae [stage 25–31 (Gosner's table)] without previous contact with contaminants were used as sentinel species. Stock animals were kept in glass aquaria with running TW.

Before the experiments, tadpoles were acclimated to laboratory conditions at constant temperature (21 $\pm$ 1 °C). According to the sampling season, the photoperiod was adjusted to 16:8-, 12:12-, or 8:16-h light-to-dark cycles. During acclimation, animals were fed ad libitum once a day with commercial fish pellets of the following composition (in g 100 g$^{-1}$): 12.7 lipids, 39.7 protein, 28.6 carbohydrates, 7.8 humidity, and 11.2 ash. Tadpoles were then transferred to glass aquaria with TW, aeration, and under a constant recirculation regime (Cole Palmer System Peristaltic pump model no. 7553-85, Cole Palmer Instrument Co. 625 East Bunker Court, Vernon Hills, IL, USA) at a rate of 17–22 mL min$^{-1}$. The density of organisms in the aquaria was 1–2 g L$^{-1}$. The length (cm) of the tadpoles ($N = 124$) was 7.0 $\pm$ 0.1, and the body weight (g) was 3.4 $\pm$ 0.1 (mean $\pm$ SEM).

### Description of the Study Area

Samples were taken from the lake formed at the dam (D = 34°40′16.47″S; 58°52′46.19″W), and 18 km downstream on the main course (M = 34°36′25.37″S; 58°43′01.37″W) (Fig. 1). D was considered the reference site representative of the upstream condition of the river; in contrast, M is a heavily populated area with precarious settlements on its margins, where sewage goes enters the soil or groundwater.

### Sampling of River Water: Environmental Conditions

Samplings of surface water (15- to 20-cm depth) were performed in August 2009 (winter), November 2009 (spring), February 2010 (summer), and May 2010 (autumn). Total

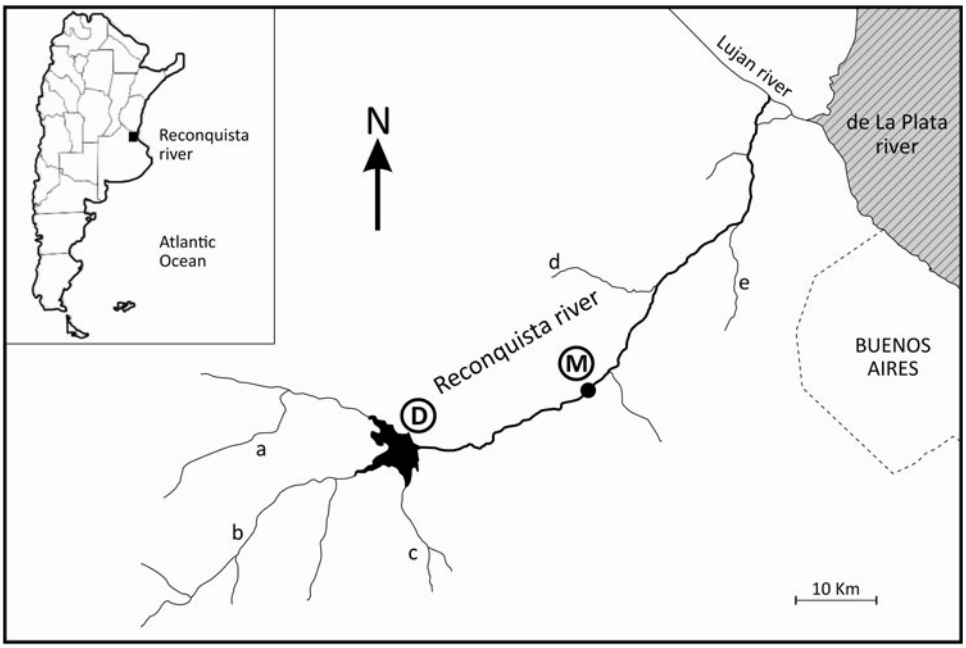


**Fig. 1** Geographic location of Reconquista River and sampling sites. D upstream at San Francisco Lake, at the dam, and at M 18 km downstream in the main course. *a* La Choza stream. *b* Durazno stream. *c* La Horqueta stream. *d* Las Catonas stream. *e* Morón stream

rainfall in the watershed during the sampling period was 1,260 mm, whereas during the week before each sampling it was 16, 1, 123.5, and 0 mm, respectively.

Water samples for physicochemical analyses were conditioned in clean bottles and immediately carried to the laboratory in coolers containing ice and stored (3–5 days) at 4–8 °C until analyzed. A sample of 100 L for bioassays was simultaneously collected in each site in plastic containers and stored at 4–8 °C. Samples for heavy-metal determinations were taken in plastic bottles and kept acidified with $HNO_3$ (pH $\leq$ 2), whereas those for pesticide determinations were collected in amber-colored glass bottles.

## Toxicity Bioassays: Experimental Design

After acclimation in the laboratory for 7 days, two groups of tadpoles were exposed to the river water samples (D and M) for 96 h; a third group of animals was exposed to control TW; aquaria with 10–15 tadpoles each had aeration and constant flow. Results obtained from samples at D were compared with those obtained at M and TW. TW was chosen for the control because no part of the river is devoid of pollutants (Salibián 2006).

## Physicochemical Analysis of the Samples

### *Field Determinations*

Temperature, pH, and conductivity were measured with a Hanna portable sensor and an Orion EA 940 pH meter.

### *Laboratory Determinations*

Alkalinity and chloride concentration were quantified by titrimetry ($H_2SO_4$ and $AgNO_3$). The concentration of ammonium ($NH_4^+$), nitrites ($NO_2^-$), nitrates ($NO_3^-$), soluble-reactive phosphorous, and chemical oxygen demand (COD) were performed by colorimetric methods. Dissolved oxygen (DO) was estimated using the titrimetic procedure (Winkler method); 5-day biochemical oxygen demand ($BOD_5$) was determined as the difference between initial and 5-day oxygen concentration. All of the analyses were performed in triplicate according to the recommendations of the APHA-AWWA WEF (2005).

## Pesticides

Screening of organochlorine and organophosphate pesticides was performed on river samples collected in 2010 by high-resolution capillary gas chromatography (Hewlett Packard 61530 Plus A6890) equipped with appropriate capture detectors (electron capture, flame photometric, and nitrogen phosphorous). The screening included the following pesticides:

1. 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.
2. Organophosphates: bromophos, chlorfenviphos, chlorpyriphos, coumaphos, diazinon, ethylbromophos, ethion, fenitrothion, malathion, and methylparathion. The

detection limit was 0.03 μg $L^{-1}$ for organochlorines and 0.02 μg $L^{-1}$ for organophosphates.

## Heavy Metals

Concentrations of arsenic (As), zinc (Zn), copper (Cu), chromium (Cr), cadmium (Cd), and lead (Pb) in river water and TW samples were measured using atomic absorption spectrophotometry. As, Cr, Cd, and Pb content were determined in a Shimadzu model 6700 instrument; Zn and Cu were determined in a model 6800 instrument with both a graphite furnace atomizer (model GFA 7000) and an autosampler (ASC 6000); and Zn content was determined in equipment coupled with air-acetylene flame. In all cases, hollow lamps (Hamamatsu Photonics) were used. Certified standards were used (1.000 g metal $L^{-1}$ CertiPUR; Merck, Darmstadt, Germany). Results are expressed as means of two readings; the detection limit was in the range of 0.5–1.0 μg $L^{-1}$.

## WQIs

Two WQIs were calculated. The first (Berón 1984) was an index of organic pollution based on the following parameters: DO, $Cl^-$, $BOD_5$, and $NH_4^+$. The second was a modification of that proposed by Pesce and Wunderlin (2000) based on temperature, hardness, $Cl^-$, DO, $NH_4^+$, $NO_2^-$, $NO_3^-$, soluble-reactive phosphorus, and $BOD_5$. These WQIs are unitless and range from 0 (highly polluted) to 10 (high purity).

## Morphological Parameters

After the exposure period, animals were anesthetized by placing them in ice water and then weighed (g). Their length (cm) was measured for calculation of the condition factor [CF = 100 × body weight/(total length)$^3$]. Animals were sacrified by an incision behind the operculum and the liver of each animal was removed and weighed (g) for calculation of the hepatosomatic index (HSI = 100 × liver weight/body weight).

## Biomarkers

The whole brain, liver, and gills were excised and stored in liquid nitrogen for further enzymatic analyses. Preparation of homogenates was performed in accordance with Ossana et al. (2010a). The following biomarkers were determined in the supernatants.

### *AChE*

AChE (EC 3.1.1.7) activity was determined according to the method of Ellman et al. (1961) by measuring the increase in absorbance of the sample at 25 °C at 412 nm for 2 min in the presence of 1 mM acetylthiocholine as substrate and 0.1 mM of 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB).

### *CAT*

CAT (EC 1.11.1.6) activity was measured according to the method of Baudhuin et al. (1964). The reaction mixture consisted of 0.05 M of phosphate $NaH_2PO_4$ buffer (pH 7.2), 17.8 mM of $H_2O_2$, and 10–20 μL of the postmitochondrial fraction (PMF) of the homogenate in a final volume of 1.5 mL. Changes in absorbance at 25 °C were recorded at 240 nm for 60 s.

### *GST*

GST (EC 2.5.1.18) activity was determined according to the method of Habig et al. (1974). The reaction mixture consisted of 0.1 M of $NaH_2PO_4$ buffer (pH 6.5), 10 mM of GSH 20 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 10 μL of PMF in a final volume of 1.3 mL. The change in absorbance at 25 °C was recorded at 340 nm for 2 min.

### *SOD*

SOD (EC 1.15.1.1) activity was measured only in liver tissue according to the method of McCord and Fridovich (1969). The reaction mixture consisted of 50 mM of $K_2HPO_4/KH_2PO_4$ buffer (pH 7.8), 100 μM of ethylene diamine tetraacetic acid, 50 μM of xanthine, and 10 μM of cytochrome c. The change in absorbance was recorded at 550 nm at 25 °C. Activity is reported as the ability to inhibit 50 % of cytochrome c decrease.

### *GSH*

GSH content was determined by the method of Ellman (1959). PMF was treated with 10 % trichloroacetic acid in a 1:1 ratio and centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was used for GSH estimation. The assay mixture contained 100 μL of supernatant and 1 mL of DTNB. Thiolate anion formation was determined at room temperature at 412 nm for 15 min against a GSH standard curve.

### *LPO*

LPO was determined in liver by measuring the concentration of 2-thiobarbituric acid reactive substances (TBARS) according to the method of Oakes and van der Kraak (2003). Aliquots of liver were homogenized (using a Teflon-glass homogenizer at 3,000 rpm) in a 1:10 w/v ratio

with 1.15 % KCl containing 35 mM of butylated hydroxytoluene (BHT). The homogenate was added to the reaction mixture containing 200 µL of 8.1 % sodium dodecyl sulfate, 1.5 mL of 20 % acetic acid adjusted to pH 3.5, 1.5 mL of 0.8 % TBA, 200 µL of 1.407 mM BHT, and distilled water to a final volume of 4 mL. The mixture was heated in a 95 °C water bath for 60 min. After cooling, 1 mL of distilled water and 5 mL of *n*-butanol were added and vortexed. After centrifugation at 5,000 rpm for 10 min at 15 °C, the immiscible organic layer was removed and its fluorescence measured by excitation at 515 nm (slit width 10 nm) on a Shimadzu RF-540 Fluorometer whose emission peak was at 553 nm (slit width 5 nm). The concentration of lipid peroxides was calculated from the fluorescence at 553 nm using tetramethoxypropane as external standard.

*Proteins*

Tissue protein content was determined according to Lowry et al. (1951), using bovine serum albumin as standard. All measurements of biomarkers were performed in triplicate, and calculations were made on the basis of average percentage normalized values. Enzymatic activities were calculated in terms of protein content of the sample and expressed as percentage relative to the control.

*Micronucleus Test*

At the end of exposure, blood cells of each group were obtained by cardiac puncture with heparinized syringes from the heart of animals that were previously anesthetized. Two blood smears for each larva were prepared on clean slides and fixed with ethanol 96 % for 10 min, air-dried, and stained with Acridine Orange (Sigma-Aldrich, CAS No. 158550) according to the method described by Schmid (1975), Ueda et al. (1992). For micronuclei analysis, erythrocytes were examined using a Zeiss HBO50 epifluorescence microscope at 400× magnification. For each tadpole, 1,000 erythrocytes from each slide were examined; results are expressed as number of cells with micronuclei/2,000 cells. Micronucleus (MN) was performed according to the criteria of Grisolia (2002).

A group of animals were exposed to TW [negative control (NC)], and a fourth group of tadpoles was exposed to TW containing 40 mg/L of cyclophosphamide monohydrate (Sigma-Aldrich, CAS No. 6055-19-2) [positive control (PC)].

*Chemicals*

All reagents were obtained from Sigma-Aldrich; solutions were made using Milli-Q water. The enzymatic activities of the supernatant were determined in a Metrolab 1700 UV–Vis spectrophotometer.

Statistical Analyses: Chemometric Analyses

Data on biomarkers are reported as mean ± SEM relative to the control. Normality and homoscedasticity of the data were checked by the Kolmogorov–Smirnov and Bartlett tests, respectively. One way analysis of variance (ANOVA) was performed followed by multiple comparison test (Tukey). Kruskal–Wallis test was used for the cases that did not fulfill the required conditions (Zar 2010). The significance level was set at $p < 0.05$.

To examine the discriminating power of the measured biomarkers, principal component analysis (PCA) was performed (Pla 1986; Guisande González et al. 2006). A data matrix was constructed with 13 biomarkers as independent variables. Variables measured at both sites of the river and in the TW control were included as absolute values. In this analysis, normality was checked by Kolmogorov–Smirnov test, and data were standardized. ANOVAs were performed using GraphPad InStat (GraphPad software Inc. version 3.01, La Jolla, USA), and PCAs were performed using Statistica 7 (StatSoft, Inc 2004, Tulsa, USA) for windows.

## Results

Chemical Profile and Water-Quality Indices of the Samples

The results of the analyses are listed in Table 1. pH was stable (6–8.2); hardness and alkalinity were greater at the river site M than at the dam (D). DO was low (<5.5 mg $L^{-1}$) in several river samples. Other domestic pollution indicators (e.g., chlorides, ammonia, soluble-reactive phosphorous, $BOD_5$, and COD) were generally higher in the river samples than in the TW. Concentrations of chlorides and alkalinity in M were always twice the value of that those found at D. In autumn and spring, ammonia levels were high (7.7 and 5.8 mg $L^{-1}$, respectively), whereas at D they were as low as 0.1 mg $L^{-1}$.

As to $BOD_5$, in all samples of M it was >10 mg $O_2$ $L^{-1}$, indicating an increased presence of organic matter. The COD/$BOD_5$ ratio showed a high contribution of nonbiodegradable organic matter, especially at D, where values were close to >10. In M, the values for that ratio were close to 4, indicating that the organic matter might be moderately degradable.

Pesticide concentrations were always lower than the detection limit of the analytical techniques; therefore, they were not included in Table 1. Calculation of the two WQIs presented values between 3 and 5 in M samples indicating pollution, whereas in D values of moderated pollution

**Table 1** Physicochemical parameters and WQIs of Reconquista River water and TW

| Parameters | Unit | Winter 2009 | | Spring 2009 | | Summer 2010 | | Autumn 2010 | | TW[f] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | D | M | D | M | D | M | D | M | |
| pH[a] | | 6.3 | 6 | 8 | 7.6 | 6.9 | 7.4 | 8.2 | 7.2 | 8.2–8.8 |
| Temperature[a] | °C | 16 | 16 | 24 | 23 | 25 | 26 | 11 | 14 | 18–20 |
| Conductivity[a] | $\mu S\ cm^{-1}$ | 644 | 1,342 | 400 | 760 | 130 | 310 | 820 | 970 | 809–1150 |
| Hardness[a] | mg $CaCO_3\ L^{-1}$ | 60 | 150 | 107 | 104 | 30 | 66 | 90 | 130 | 70–90 |
| Alkalinity[a] | mg $CaCO_3\ L^{-1}$ | 253 | 711 | NM | NM | 379 | 878 | 346 | 614 | 350–450 |
| Chlorides[a] | mg $Cl^-\ L^{-1}$ | 30 | 63 | 25 | 57 | 5 | 19 | 42 | 59 | 20–30 |
| DO[a] | mg $O_2\ L^{-1}$ | 7 | 2 | 5.5 | 4 | 0.5 | 2.5 | 7.5 | 8 | 8–10 |
| Ammonium[a] | mg N-$NH_4^+\ L^{-1}$ | 0 | 1 | 0.1 | 5.8 | 0.2 | 0.2 | 0.1 | 7.7 | 0.3–0.7 |
| Nitrites[a] | mg N-$NO_2^-\ L^{-1}$ | 0.02 | 0.14 | 0.02 | 0.36 | 0.02 | 0.1 | 0 | 0.2 | 1.5–1.7 |
| Nitrates[a] | mg N-$NO_3^-\ L^{-1}$ | 0.6 | 0.2 | 1.3 | 0.9 | 0.06 | 0.80 | 1.6 | 1.4 | 6–10 |
| Soluble-reactive phosphorus[a] | mg P-$PO_4^{-3}\ L^{-1}$ | 0.5 | 2.2 | 0.6 | 1.5 | 0.4 | 0.5 | 0.6 | 1.6 | 0.12–0.35 |
| $BOD_5$[a] | mg $O_2\ L^{-1}$ | 8 | 28 | 11 | 11 | 4 | 16 | 6 | 13 | 1–2 |
| COD[a] | mg $O_2\ L^{-1}$ | NM | NM | NM | NM | 52 | 59 | 53 | 48 | 0 |
| $COD/BOD_5$ | | NM | NM | NM | NM | 13 | 3.7 | 8.8 | 3.7 | 0 |
| Heavy metals[b] | $\mu g\ L^{-1}$ | | | | | | | | | |
| As[c] | | **26**[e] | **29** | 13 | **16** | 7 | **16** | **24** | **25** | **40** |
| Zn[d] | | < 10 | 54 | 12 | 12 | **110** | 24 | **55** | <10 | 15 |
| Cu[c] | | **5** | 7 | **12** | **8** | **3** | 3 | 4 | 8 | **10** |
| Cr[c] | | **3** | **7** | <2 | **13** | <2 | <2 | **6** | <2 | <2 |
| Cd[c] | | **2** | **2** | <2 | <2 | <1 | <1 | < 5 | <5 | <5 |
| Pb[c] | | <0.5 | <0.5 | <1 | <1 | <0.5 | <0.5 | **6** | **1** | <0.5 |
| WQIs | | | | | | | | | | |
| Berón (1984) | | 8 | 4 | 6.5 | 3 | 4 | 5 | 7 | 4 | 9 |
| Pesce and Wunderlin (2000) | | 7 | 4 | 6 | 5 | 7 | 5 | 7 | 5 | 9 |

Data of river samples were taken at D and at M

[a] Means of triplicate measurements

[b] Means of duplicate measurements

[c] *GFAAS* graphite furnace atomizer absorption spectrophotometry

[d] *AAF* air acetylene flame, *NM* not measured

[e] Heavy-metal concentrations greater than local guidelines for aquatic life protection are shown in bold text

[f] TW data are presented as ranges

between 6 and 8 were found. In contrast, both indices for TW were 9.

It should be noted that before summer sampling, there were heavy rains, which provoked dilution in some parameters of the samples (e.g., nitrogen compounds, phosphorous, chlorides). It is interesting to remember that metal toxicity is influenced by the ambient chemistry, which affects their speciation and its impact on their bioavailability (Cooney 1995). It is known that the ecotoxicity of metals in particular is inversely related to hardness: As it increases, metal toxicity decreases. In our case, hardness in D varied between 30 and 90 mg $CaCO_3\ L^{-1}$. However, in M, hardness varied between 60 and 150 mg $CaCO_3\ L^{-1}$.

Most of the heavy metals measured was found frequently to be greater than the local guidelines for the protection of the aquatic life. Table 2 list the allowed threshold values of heavy metals for aquatic life protection of Argentina legislation compared with those of Canada and the United States. It can be seen that Argentinean law is more permissive.

## Biomarkers

The results of brain, gill, and liver enzyme activities, as well as GSH and TBARS concentrations and morphological parameters, are listed in Table 3.

**Table 2** WQIs for the protection of aquatic life for heavy metals in freshwater for Argentina, Canada, and the United States

| Parameter ($\mu g\ L^{-1}$) | Argentina | | Canada | United States (USEPA) |
|---|---|---|---|---|
| | National Law 24051 | Subsecretary of water resources | | |
| As | 50 | ≤15 | 5 | 150 |
| Zn | 30 | ≤41.4 | 30 | 120 |
| Cu | 2 | ≤6.2 | 2–3[a] | – |
| Cr | 2 | ≤2.5 | 8.9–1[b] | 74–11[b] |
| Cd | 0.2 | ≤0.11 | 0.017 | 0.25 |
| Pb | 1–4[c] | ≤1.59 | 1–4[b] | 2.5 |

*USEPA* United States Environmental Protection Agency

[a] 2 $\mu g\ L^{-1}$ at a water hardness of 0–120 mg of $CaCO_3\ L^{-1}$; 3 $\mu g\ L^{-1}$ at a water hardness of 120–180 mg of $CaCO_3\ L^{-1}$

[b] Cr trivalent and hexavalent, respectively

[c] 1 $\mu g\ L^{-1}$ at a water hardness of 0–60 mg of $CaCO_3\ L^{-1}$; 2 $\mu g\ L^{-1}$ at a water hardness of 60–120 mg of $CaCO_3\ L^{-1}$; and 4 $\mu g\ L^{-1}$ at a water hardness of 120–180 mg of $CaCO_3\ L^{-1}$

*AChE*

In some cases, AChE activity was inhibited with respect to the control mainly in animals exposed to samples from M; these differences were statistically significant during autumn 2010.

*CAT*

CAT activity in gill was significantly decreased for both sites (D and M) in three of the samplings (winter, spring, and autumn). In liver, values were stable and varied near control values with the exception of autumn 2010.

*GST*

Gill showed high variability of GST: It increased in animals exposed to the sample from D during winter 2009, whereas it was decreased 50 % in spring and then increased 40 % in summer for animals exposed to water from M. Liver values were stable and varied near those of the control.

*GSH*

GSH concentration in gill appeared as the biomarker with a greater increase compared with the control (at both sites in spring). In liver, GSH also increased notably with respect to the control in spring and summer for animals exposed to D, whereas for animals exposed to M, values were only slightly greater than those of the control (except for the sampling in the autumn, which decreased by 40 %).

*SOD-TBARS*

SOD activity was statistically higher in M for samples taken during winter and summer, whereas lipidic peroxidation showed no statistically significant changes; its values were close to those obtained in larval controls.

*Proteins*

Significant protein variation was found in brain. For gill and liver, total protein content showed a tendency toward stability and varied at values near those of the control.

Morphological Parameters

CF remained stable with values identical to those of the control. The HSI increased in winter at site M and decreased in summer at both sites (D and M).

Genetic Biomarkers

Comparing the amount of erythrocytes with MN found in peripheral blood of larvae exposed to environmental sampling, none of the samples presented significant differences between sites (D and M), and, in general, values were similar to those of PC. In the summer sampling, MN frequency at D and M was greater than at NC, but this was not statistically significant. This result might be a consequence of the heavy rains that occurred before sampling. The number of MN in larvae exposed to PC showed a significant increase (260 %) compared with the basal values found in NC (Fig. 2).

*Chemometrics*

In the PCA performed using the biomarkers, the first two factors explained the 51.21 % of the total variance (Fig. 3a). Factor 1 (eigenvalue = 3.66) is positively charged by GST in liver and gill (0.79 and 0.82), MN frequency (−0.64), and protein content in liver and gills (−0.69 and −0.65). Factor 2 (eigenvalue = 2.99) is positively charged by GSH load in liver (0.77) as well as AChE brain activity (−0.68) and protein content (0.67). In Fig. 3b, a discrimination by sampling season can be noted.

## Discussion

This article presents the results obtained from a year-long monitoring program that included analyzing the chemical composition of the water and evaluating a battery of physiological, morphological, and genetic biomarkers in premetamorphic larvae of *L. catesbeianus* exposed to

**Table 3** Biomarkers and morphological parameters of *L. catesbeianus* tadpoles exposed for 96 h to water samples from Reconquista River

| Tissue | Biomarkers | Winter 2009 | | Spring 2009 | | Summer 2010 | | Autumn 2010 | |
|---|---|---|---|---|---|---|---|---|---|
| | | D | M | D | M | D | M | D | M |
| Brain | AchE | 107 ± 9# | 74 ± 10 | 79.5 ± 12 | 80 ± 7 | 122 ± 6 | 98.5 ± 5 | 118 ± 7 | 72 ± 5#* |
| | Protein | 101 ± 9 | 96 ± 6 | 112 ± 4 | 111 ± 2 | 161 ± 6.5#* | 57 ± 6* | 72.8 ± 10 | 109 ± 7# |
| Gills | CAT | 74.5 ± 6.2* | 65.7 ± 8.8* | 70.7 ± 9.5* | 77.3 ± 4.8* | 107.5 ± 11.7 | 149.4 ± 6.7#* | 54 ± 7* | 81 ± 7#* |
| | GST | 148.1 ± 6.8#* | 100.6 ± 4.2 | 101 ± 7.5 | 51.1 ± 4.5#* | 117.1 ± 7.9 | 141.3 ± 9.1* | 101.2 ± 7.1 | 98.8 ± 6.5 |
| | GSH | 85.5 ± 16.6 | 56.5 ± 22.6 | 567.8 ± 7.3#* | 454.6 ± 3* | 85.5 ± 26.1 | 81.5 ± 12 | 74.2 ± 7 | 70.4 ± 26.6 |
| | Protein | 115.6 ± 4.2 | 100.2 ± 7.3 | 103.2 ± 5.4 | 110.4 ± 5.9 | 77.8 ± 7.4* | 80.5 ± 6.7* | 100 ± 3.9 | 107.5 ± 7.6 |
| Liver | CAT | 107.8 ± 5.8 | 101.4 ± 4.4 | 80.4 ± 10.2 | 88.2 ± 8.7 | 110.2 ± 8.2 | 110.2 ± 13.5 | 94.8 ± 11.9 | 135.8 ± 12.1* |
| | GST | 125 ± 9.2 | 104.7 ± 9.5 | 80.7 ± 16.2 | 86 ± 5 | 111.1 ± 6.6 | 142.9 ± 8.1* | 103.1 ± 6 | 101.9 ± 11.8 |
| | GSH | 98 ± 8.9 | 108.1 ± 11.2 | 316.9 ± 26.8#* | 163.1 ± 11.9 | 225.2 ± 16.7* | 134.8 ± 12.1 | 118.3 ± 15.3 | 28.3 ± 10.3#* |
| | SOD | 91 ± 12.3 | 158 ± 11.9#* | NM | 88 ± 13.3 | 108.9 ± 18.9 | 159.9 ± 18.3* | 109.8 ± 21.6 | 87.3 ± 19.2 |
| | TBARS | NM | NM | 130.7 ± 28.7 | 104.4 ± 12.1 | NM | 122.5 ± 12.2 | 81.2 ± 23.1 | 123.3 ± 16.8 |
| | Protein | 77 ± 5.9* | 70.3 ± 7.6* | 115.2 ± 3.9 | 114.8 ± 5.5 | 100.1 ± 6.5 | 90.8 ± 5.4 | 123.8 ± 8.2 | 112.3 ± 4 |
| | HSI | 104 ± 5 | 165 ± 6 #* | 95.5 ± 7 | 96 ± 5 | 67.3 ± 5* | 77 ± 5.6* | 86.4 ± 3.4 | 93 ± 5.4 |
| | FC | 112 ± 3.2 | 111.7 ± 6.2 | 105 ± 3.6 | 97 ± 3.7 | 104 ± 2 | 108 ± 4 | 104.4 ± 4 | 106.4 ± 4 |

*NM* not measured

*Significant differences compared with the control

#Significant differences between tadpoles exposed to D and M water samples; values are presented as percentage relative to the control (mean ± SEM); $n = 10–15$

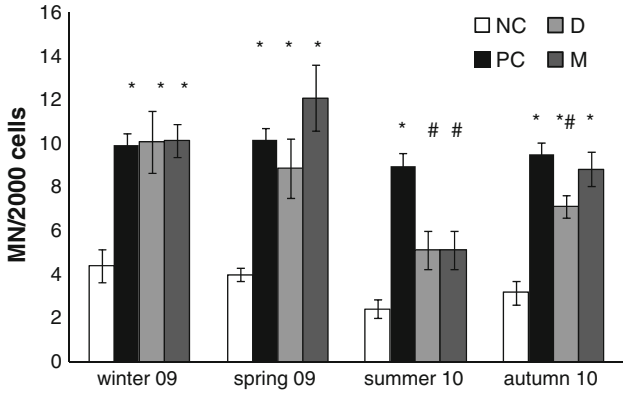


**Fig. 2** Frequency of micronuclei in peripheral erythrocytes from *L. catesbeianus* tadpoles exposed to NC, PC, and water from D and M sites of Reconquista River. Data as mean ± SEM; $N = 7–10$. *Significant differences compared with NC (TW). #Significant differences compared with PC (cyclophosphamide)

samples of the Reconquista River, which is the second most polluted river in Argentina.

Middle Reconquista River basin has at its margins a high urban–industrial concentration and shows a high level of environmental stress of anthropogenic origin. The toxicological quality of its surface water has been previously monitored throughout its course by several researchers at our laboratory (Topalián et al. 1999; Olguín et al. 2004; de la Torre et al. 2005, 2007; Salibián 2006).

Samples from D are representative of the water quality at the Roggero Dam, which is formed by the contribution of the La Horqueta, Durazno, and La Choza streams, at the river source. These samples were used as the control for comparison with those taken at the middle course of Reconquista River (M), which allowed evaluation of changes that had occurred up to that point, which lies ~18 km downstream. It should be noted that La Choza stream presents a considerable load of organic pollution produced by the discharges from a wastewater treatment plant and from a small industrial park upstream from the dam. At this site there are, among others, an agrochemical industry and a poultry farming settlement which generates effluents, which are also discharged into the stream. Basílico (2008), Vilches et al. (2011) found chemical and biological indicators of significant deterioration of the water quality in that stream.

According to Sadañiowski (2003), the reservoir formed by the dam would act as a depuration lagoon for the organic discharges from the upper basin, which drains through the streams. DO values were between 5 and 7 mg $O_2$ $L^{-1}$, whereas $BOD_5$ corresponded with the condition of a polluted water (>8 mg $O_2$ $L^{-1}$). Values found for $BOD_5$ and COD indicated the presence of significantly high levels of nondegradable organic matter with variable oxygen concentrations.

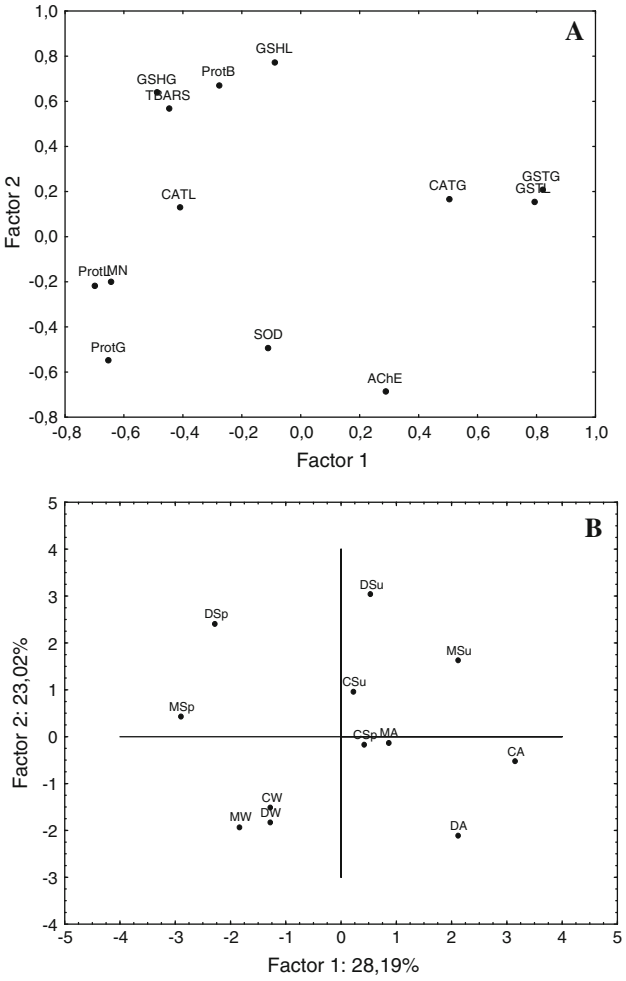


**Fig. 3** PCA plot in a plane formed by the two first components. **a** Variable biomarkers (AChE, SOD, TBARS, Prot (protein); CAT, GST, and GSH) in liver (L), gill (G), and brain (B). **b** Seasonal separation of the data based on the biomarker's responses: *C* control, *D* dam (upstream river), *M* downstream river, *Su* summer, *A* autumn, *Sp* spring, *W* winter

As for M, there was a notable decrease in water quality with respect to D, which is evident from the low DO concentration and the high $BOD_5$ levels: the $COD/BOD_5$ ratio was 3.7 (Table 1), which indicates that the organic matter in the water is moderately degradable.

Concentration of chlorides, phosphates and inorganic nitrogen compounds, alkalinity, and conductivity were high in M (doubled in almost all samples with respect to values found at D), and ammonia was frequently 5–7 times higher.

In many cases, the heavy metals concentrations were high, greater than the guidelines of the local legislation for aquatic life protection. In general, the guidelines of Canada appear to be more restrictive compared with Argentinean and United States guidelines. Before summer 2010 sampling, there were heavy rains. The dilution effect due to precipitation was evidenced by the magnitude of many

parameters, whereas others suffered minor modifications with respect to previous samplings.

It is important to note that the values of WQIs confirmed a state of high pollution at site M (Table 1); at D they were near 7, indicating a better water quality (Avila Perez et al. 2011). This shows the convenience of devise-monitoring programs integrating chemical and biochemical parameters. Frequency of MN found in peripheric erythrocytes of larvae exposed to environmental samples was, in most cases, significantly greater than the basal levels obtained in control animals in TW (NC) and similar to those already established for this species in previous studies (Ossana et al. 2010b; Ossana and Salibián 2013). MN frequency was similar to that of PCs, thus indicating considerable genotoxic response comparable with that provoked by the reference chemical. Furthermore, it is worth noting that there were no differences between sites in larvae regarding most of the biomarkers despite the differences in water quality shown by the WQIs. Despite the WQIs, genotoxicity was noted in both D and M samples. The possibility should be considered other compounds may have been present that might have also been genotoxic or could have enhanced the effect.

To evaluate which of all of the analyzed indicators could better explain the ecotoxicological situation of the river, multivariate analysis was performed. The use of PCA applied to environmental water samples contributes to a more accurate interpretation of the results by highlighting the parameters that allow the identification of sites with different water quality and, consequently, different ecotoxicological risks for the biota. Furthermore, it allows the integration of data of diverse origin (both biological and chemical) to produce complementary results, which can contribute to the analysis of environmental risks based on results from monitoring or environmental remediation protocols (Monferrán et al. 2011). Several investigators have applied the same or similar statistical techniques (discrimination analysis, clusters analysis) to analyze the quality of superficial water samples (Chèvre et al. 2003b; Mendiguchía et al. 2004; Kazi et al. 2009; Falfushynska et al. 2010; Li et al. 2011).

PCA, performed including all of the determined biomarkers in all samples, made it possible to identify those which turned out to be the most important: GST activity and GSH content in liver and gill, AChE in brain, erythrocyte MN, and tissue protein content (Fig. 3a). In addition, PCA showed seasonal separation (Fig. 3b). It can be seen from the data that in summer, there were increases in GST in liver and gill. In spring, there were increases in GSH in liver and in the frequency of MN (which also occurred during the winter), whereas during autumn, AChE and GSH in liver decreased in samples of M.

Our results confirm that the physicochemical analysis of the sites describe the environmental situation of the river and show that is necessary to integrate chemical data with changes registered in biomarkers, which give additional information on the water quality. It is interesting to note that Trujillo-Jiménez et al. (2011) arrived at similar conclusions. It is worth mentioning that Cazenave et al. (2009), when monitoring another periurban river in Córdoba (Argentina), did not find differences in water quality evaluated with one of the WQI used in this article, but they reported differences in biomarker responses.

The results presented in this article reflected an important condition of severe stress in the water quality of the Reconquista River, which can be seen in the responses of biomarkers *L. catesbeianus*. WQIs showed differences in water quality between D and M compared with the TW, but these differences were not observed in biomarker results, especially in the frequency of erythrocyte micronuclei. Furthermore, ours is the first genotoxicity report for the water of this river; the MN test proved to be an early and sensitive toxicity biomarker. The results of the present study corroborate the suitability of *L. catesbeianus* as a sentinel species.

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