Multibiomarker response in ten spotted live-bearer fish *Cnesterodon decemmaculatus* (Jenyns, 1842) exposed to Reconquista river water

N.A. Ossana a,b,*, B.L. Eissa b, F.G. Baudou a,c, P.M. Castañé a, S. Soloneski b,d, L. Ferrari a,e

a Department of Basic Sciences and Institute of Ecology and Sustainable Development (PRODEA-INEDES), National University of Luján, P.O. Box 221, B6700ZBA Luján, Argentina
b National Scientific and Technical Research Council (CONICET), Rivadavia 1917, C1033AAJ Buenos Aires, Argentina
c ANPCyT, Argentina
d Faculty of Natural Sciences and Museum, National University of La Plata-UNLP, Argentina
e Scientific Research Commission (CIC)-La Plata, Argentina

**Abstract**

The aim of this paper is to assess the water quality to chemical pollution at Roggero Dam, the headwater of the Reconquista river, and to perform a Cadmium (Cd) contamination pulse simulation through a wide battery of biomarkers which included: genotoxicity and enzymatic biomarker parameters on a neotropical teleost fish namely *Cnesterodon decemmaculatus*. Water samples were taken in order to determine the river’s physicochemical profile. An integrative approach was applied using a biomarker index.

The bioassay involved the use of laboratory culture adult animals, acclimatized in moderately hard water (MHW) and fed *ad libitum*. A semi-static 96 h bioassay was conducted and the experimental groups were as follows: [1] river water (Rg); [2] river water + 2 mg/L Cd (RgCd); [3] MHW + 2 mg/L Cadmium (Cd), positive metal control; [4] MHW + 5 mg/L Cyclophosphamide (positive genotoxicity control -CP); [5] MWH, negative control (NC). At the end of the exposure time fishes were sectioned and the following biomarkers were determined: 1) condition factor rate (CF); 2) for the anterior section (A) (head): glutathione (GSH) and protein (Pr) content; 3) for the body midsection (M) (viscera): Pr, GSH, Glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD). Blood samples were also taken from the fishes specimens to estimate the frequency of micronuclei (MN) as well as other nuclear abnormalities (NA).

The physicochemical profile of the river water sample indicated high Copper concentrations. CAT and SOD activity and total Pr content did not show any significant changes. GST activity decreased in fishes exposed to Rg, while GSH content decreased significantly for all treatments compared to controls in MHW. These results would seem to point to a reduction in cell defense capability as a result of the depletion antioxidants such as GSH. The NA frequency increased significantly in all treated groups while MN frequency was increased only in Cd and CP groups. Using some the biomarkers measured, a biomarker index was estimated which revealed that fishes exposed to Rg were 90% affected or highly affected, while those exposed to RgCd were 80% and Cd 68% affected or highly affected. The obtained results indicate the usefulness of the use of a battery of variables by means of the biomarker index to analyze water quality.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Bioassays respond to the bioavailable fraction of the toxicants, thus providing environmentally relevant information that complements the physicochemical data with the biological information in order to determine the consequences of exposing organisms to a polluted environment. A biomarker may be defined as any biological response by a test organism after exposure to different stress conditions. Thus, biochemical, physiological, histological, morphological and behavioral measurements used as biomarkers become sensitive tools that can be used to assess the adverse effects of several pollutants on natural populations or communities (Walker et al., 2006; van der Oost et al., 2003).

Recent studies have highlighted the importance of using suitable biomarkers at different biological response levels to provide...
an integrated relative measure of the general health status of natural environments where contaminants are usually present as complex mixtures (Maggioni et al., 2012). In this context, there is no single biomarker that can give a complete diagnosis of environmental degradation.

Cells exhibit enzymatic and non-enzymatic protective mechanisms to counteract factors causing alterations in their critical parameters, beyond a steady-state balance. These are molecules involved in detoxifying xenobiotics or their metabolites into more soluble forms that can be more easily excreted from the body (van der Oost et al., 2003). Many environmental contaminants provoke toxic effects by promoting oxidative stress (Lushchak, 2011). Reactive oxygen species (ROS) are well studied in connection with environmental chemical substances that increase production and lead to effects causing damage to the organism at cellular level. Among them, other pollutants, e.g. heavy metals, herbicides and aromatic compounds produce an imbalance due to excess ROS or oxidant in cell capacity to promote an effective antioxidant response. While organisms have antioxidant defenses against ROS of their own production, environmental pollutants alter this equilibrium leading individuals into an oxidative stress condition. Key biological molecules, notably DNA, proteins, and lipids, can all be adversely affected by ROS (Di Giulio and Meyer, 2008).

Antioxidant enzymes facilitate the removal of resulting increase ROS. For example, superoxide anion (O₂⁻) can be metabolized by superoxide dismutase (SOD) to H₂O₂. This hydrogen peroxide molecule can then be reduced to H₂O and O₂ by catalase (CAT). Also, there are a number of molecules that function as scavengers of free radicals. One of the most abundant and most important molecular antioxidant in cellular cytoplasm is reduced glutathione (GSH). GSH is a low molecular weight thiol that can react directly with ROS species, thereby detoxifying them. In addition, GST (glutathione-S-transferase) catalyzes GSH conjugation with electrophiles and facilitates xenobiotics excretion (Lushchak, 2011).

The micronucleus (MN) assay in peripheral blood erythrocytes is one of the best established in vivo and in vitro cytogenetic bioassay in the Genotoxicology field; this assay provides a convenient and reliable index of both chromosome breakage and/or whole chromosome loss (Fenech, 2000).

Among genotoxicity biomarkers, in vivo micronucleus assay is widely employed to evaluate various chemicals and polluted aquatic environments such as rivers (Arkhchipuk and Garanko, 2005). During the micronuclei assay, some authors have observed the occurrence of other nuclear abnormalities (NA) seems to be one of the most abundant and most important. Most of these NA are connected to cell division failures and cell death processes, as well as to genotoxicity and/or mutagenicity and may complement the MN scoring in routine assays for genotoxicity screening (da Silva Souza and Fontanetti, 2006; Lajmanovich et al., 2014). The NA type like lobed, bi-nucleate, kidney-shaped nuclei, etc. estimations, represent an alternative to MN testing and overcome any possible lack of sensitivity in connection with the low MN frequency found in wild fish populations (Guilherme et al., 2008).

In their natural environment organisms exhibit an integrated response to multiple pollutant contamination from their environment, in an effort to detoxify and recover their homeostatic balance. This disturbance involves different biological processes at differing organizational levels. Thus, the employ of several biomarkers can provide a better approximation for characterize the toxicity of a xenobiotic under study.

The Reconquista river is the second most contaminated watercourses in Argentina, having been the disposal point over many decades for numerous contaminating effluents of urban, agricultural, cattle-farming and industrial origin. It should be noted that 90% of industrial waste produced in the area is dumped directly into watercourses (Salibían, 2006). Several studies have been previously conducted on the Reconquista river using physicochemical water quality assessment and chronic and acute toxicity bioassays on amphibians and fish under field and laboratory conditions (i.e. Ferrari et al., 2005; de la Torre et al., 2007; Ossana et al., 2013). The water quality index (WQI) for the high basin of this river indicates slight pollution values (Rigacci et al., 2013), yet highly stressful conditions have been found in animals exposed to these waters (Ossana et al., 2013; Ossana, 2011; de la Torre et al., 2005).

_Cnestodon decemmaculatus_ (Poeciliidae, Cyprinodontiforme) is a widely distributed species in Neotropical America, densely populating a large variety of South American water-bodies including the middle stretch of the Reconquista river (Ferrari, 2015). It is a small viviparous, micro-omnivorous, benthic-pelagic, non-migratory fish found in both pristine and severely degraded habitats (Hued and Bistoni, 2005). According to Gopalakrishnan et al. (2008) to be of practical use in ecotoxicological assessment bioassays, a candidate species, should be not only sensitive to potential contaminants, but also relatively easy to collect from the field (i.e. abundant) as well as amenable to routine maintenance, culture and rearing in the laboratory (Sonna et al., 2011). Furthermore, several studies have found this species a suitable test organism in acute and chronic toxicity bioassays (de la Torre et al., 2002, Menéndez-Helman et al., 2012; Vera-Candioti et al., 2010, 2013; Mastrángelo and Ferrari, 2013). _C. decemmaculatus_ is also one of the neo-tropical fish species recommended for use in standardized monitoring bioassays (IRAM, 2008).

Cadmium (Cd) is a non-ferrous metal for which no physiological function has been demonstrated. In aquatic environments, concentrations of this metal have been increased due to anthropic activities. It is one of the most toxic metals for aquatic organisms and, though a non-essential element, follows the metabolic pathway of essential elements such as zinc, copper and calcium (Mebane, 2006). Numerous effects have been observed among aquatic vertebrates exposed to Cd, including: changes in swimming activity and abnormal swimming patterns (Eissa et al., 2010; Sloman et al., 2003), alterations in gill morphology (Ferrari et al., 2009), alterations in energy balance (Ferrari et al., 2011), increase in antioxidant enzyme activity (Almeida et al., 2002; Dabas et al., 2012), increase in micronuclei frequency (Ossana et al., 2009), etc. This metal has been proposed and used as a reference toxic element for _C. decemmaculatus_ in acute lethality bioassays on juvenile animals (de la Torre et al., 1997; Mastrángelo and Ferrari, 2013) and is currently being assessed for use as a positive control for early effect endpoints.

This study considers the use of an integrative biomarker approach to analyzing surface water effects in the headwater of the Reconquista river basin, and to perform a Cadmium (Cd) contamination pulse simulation on adult _Cnestodon decemmaculatus_. The aim of the study was to assess water quality through a wide battery of biomarkers including genotoxic, enzymatic and non-enzymatic biomarkers on a neotropical teleost fish.

2. Material and methods

2.1. Water sampling

Water samples were taken from the body of water formed at the Roggero dam (D: 34°40′16.47″ S; 58°52′46.19″ W) (Fig. 1), located in the boundary area between the upper and middle basins of the Reconquista river. Although built to reduce overflow due to flooding, the reservoir could be considered a depuration system.
for transported material and, therefore, for water quality. This is particularly significant since water from La Choza, La Horqueta and Durazno streams enters the reservoir transporting sewage water among other contaminants (Rigacci et al., 2013).

Water samples were taken in spring 2013. No rainfall was recorded at the time the sample was taken or during the 9 days prior to the sampling, with a total rainfall of 123 mm recorded during the month of the sampling.

Surface water samples for physicochemical analyses were conditioned in clean bottles and immediately taken to the laboratory in coolers containing ice, and stored (3–5 days) at 4–8°C until analyzed. Samples for heavy metal determinations were taken in plastic bottles and kept acidified with HNO₃ (pH < 2), while those for pesticide determinations were collected in amber-colored glass bottles.

A surface water sample of 20 L for bioassays was simultaneously collected in plastic containers, and stored at 4–8°C until used on the first 48 h after sampling.

2.2. Test organisms

Cnesterodon decemmaculatus were collected from own outdoor culture and transferred to indoor culture for a holding time with dechlorinated tap water and controlled temperature (21±1°C) and photoperiod (16 h L/8 h D). The 15-day acclimation period was conducted in glass aquaria (14 × 14 × 20 cm), each containing ten fishes in 2 L (load < 1 g/L) of MHW (Moderate Hard Water) having the following composition (mg/L): NaHCO₃, 96; CaSO₄·2H₂O, 60; MgSO₄, 60; KCl, 4; pH, 7.4–7.8; hardness, 80–100 mg CO₃Ca/L (Weber, 1993). During the holding and acclimation period the fishes were daily fed ad libitum with crushed fish food (TetraFin®). Aquaria were placed in a permanently aerated incubation chamber, at the same temperature and photoperiod, and were kept in these conditions until the assays had been completed. The conditions of photoperiod, temperature and animals load (g/L) were the same throughout the bioassay.

One hundred male and female animals of average weight 97.85 ± 6.10 (g) and length 23.72 ± 0.35 (mm) (Media ± SEM) were used.

2.3. Toxicity bioassays. Experimental design

After a 15-day acclimation period, the fish were exposed in duplicate to the following treatments for 96 h: 1) river water samples (Rg); 2) river sample with a Cd contamination pulse (2 mg/L Cd) (RgCd), 3) MHW (negative Controls-NC); 4) MHW with a Cd contamination pulse at the same concentration (2 mg/L) (positive metal control-Cd); 5) MHW with 5 mg/L of Cyclophosphamide (CP), a reference toxic element for genotoxicity assays (MN positive control) with additional NC. Ten individuals were placed in each aquarium. During the assay, the aquaria water medium (2 L) was renewed every 48 h to each treatment. The photoperiod, temperature, aquaria size and animals load were the same as during the acclimation period.

During the assay several physicochemical parameters were controlled daily in the aquaria: hardness, DO, pH, conductivity and Cd concentration.

Fig. 2 shows a schematic flow chart of the experimental design used.

2.4. Physicochemical analysis of the samples

Field determinations. An electrochemical portable device (HQD Field case Hach) was used for in situ measurements of temperature, pH and conductivity.

Laboratory determinations. Alkalinity and chloride concentration were quantified by titration (H₂SO₄ and AgNO₃). Ammonium (NH₄⁺), nitrates (NO₃⁻), and soluble reactive phosphorous (SRP) from filtered water (0.45 μm) and Chemical Oxygen Demand (COD) concentrations were evaluated by colorimetry. Dissolved oxygen (DO) was estimated by titration (Winkler method) and 5-day Biochemical Oxygen Demand (BOD₅) was indicative of oxygen consumption over 5 days. All the analyses were performed in triplicate following APHA (2005) recommendations.

Two water quality indexes (WQIs) were calculated. The first (WQIa) (Berón, 1984) is an organic pollution index based on the following parameters: DO, Cl⁻, BOD₅, and NH₄⁺. This is a domestic contamination indicator. The second (WQIb) (Lacoste and Collasius, 1995) is an indicator of pollution of industrial origin based on:
DO, COD, Pb, As and Cu. These WQIs are unitless and range from 0 (highly polluted) to 10 (high purity).

2.4.1. Pesticides

Screening of organochlorine and organophosphate pesticides was performed on river samples by high-resolution capillary gas chromatography (Hewlett Packard 61,530 Plus A6890) equipped with appropriate capture detectors (electron capture, flame photometric, and nitrogen phosphorous). Screening included the following pesticides:

Organochlorines: aldrin, DDT and their metabolites, dieldrin, α- and β-endosulfan, endrin, heptachlor, heptachlor epoxide, hexachlorobenzene, α- and β-hexachlorocyclohexane.

Organophosphates: chlorfenviphos, chlorpyriphos, coumaphos, diazinon, ethylbromophos, ethion, fentrothion, malathion and methyliparathion. Detection limit was 0.03 μg/L for organochlorines and 0.02 μg/L for organophosphates.

2.4.2. Heavy metals

Arsenic (As), zinc (Zn), copper (Cu), chromium (Cr), cadmium (Cd), and lead (Pb) concentrations in the river sample and Cd concentration in all samples and MHW (controls) were measured using a Perkin Elmer analyst 200 at absorption spectrophotometer. Arsenic content was determined by atomic absorption using a FIAS 100 model photometer. Arsenic content was determined by atomic absorption spectroscopy, and nitrogen phosphorous.

2.5. Biological parameters

After the exposure period, animals were anesthetized by placing them in ice water. Then they were weighed (g) and their length (cm) was measured to calculate the condition factor [CF = 100 \times \text{body weight}/(\text{total length})^3].

Fishes were sacrificed by incision behind the opercula, and one drop of blood was taken to smear onto precleaned slides for MN detection (see above). Then, the fishes were subsequently sectioned in three parts for biomarker determination (Menéndez-Helman et al., 2012) as follows: the anterior body section (A) corresponding to the whole head (brain and gills); the body midsection up to the anus (M), corresponding to the visceral tissues and post-anal section. The A and M sections were excised and stored in freeze conditions (−80 °C) for further enzymatic analyses.

2.5.1. Biomarkers

Post-mitochondrial supernatant (PMS) was prepared from a 1:8 w/v homogenate of tissues in a buffer containing 0.1 M NaH2PO4, 0.15 M KCl, 1 mM EDTA, 1 mM DTT and 10% v/v glycerol (pH 8) using a glass/Teflon homogenizer on ice at 3000 rpm and 20 strokes. The final PMS homogenate was centrifuged (Eppendorf 5810 R centrifuge) at 10,000 \times g for 10 min at 4 °C. The following biomarkers were determined in the supernatants:

Catalase (CAT) (EC 1.11.1.6) activity was measured in the M section following the method proposed by Baudhuin et al. (1964). The reaction mixture consisted of 0.05 M phosphate NaH2PO4 buffer (pH 7.2), 17.8 mM H2O2, and 10–20 μl of PMS homogenate, in a final volume of 1.5 ml. Changes in absorbance at 25 °C were recorded at 240 nm for 60 s. Enzyme activity was expressed in μmol H2O2 consumed min⁻¹ mg⁻¹ protein.

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured only in the M section following the method suggested by McCord and Fridovich (1969). The reaction mixture consisted of 50 mM of K2HPO4/KH2PO4 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 the reduction in cytochrome c. Enzyme activity was expressed as Units. mg protein⁻¹.

Glutathione-S-transferase (GST) (EC 2.5.1.18) activity was...
determined in the M section using the method proposed by Habig et al. (1974). The reaction mixture consisted of 0.1 M NaH₂PO₄ buffer (pH 6.5), 10 mM GSH, 20 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 10 μl PMS in 1.3 ml. The change in absorbance at 25 °C was recorded at 340 nm for 2 min. Enzyme activity was expressed as μmole CDNB conjugate formed min⁻¹ mg⁻¹ protein.

Glutathione (GSH) content was determined in the A and M sections using the method described by Ellman (1959). PMS was treated with 10% trichloroacetic acid (TCA) in a 1:1 ratio and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was used for GSH estimation. The assay mixture contained 100 μl supernatant and 1 ml DTNB. Thiolute anion formation was determined at room temperature at 412 nm for 15 min against a GSH standard curve. GSH was determined as acid-soluble thiols (AST) and results were expressed as μmols of soluble thiols per gram of tissue.

Tissue Protein (Pr) content was determined for A and M sections according to Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

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

2.5.2. Micronuclei frequency

One drop of peripheral blood from each animal was smeared onto clean slides. The slides were then air dried, fixed with 100% (v/v) cold methanol (4 °C) and stained with 5% Giemsa solution. Slides were coded and blind-scored at 1000 x magnification. MN frequency was determined by analyzing 1500 mature erythrocytes from each fish as suggested previously (Vera-Candioti et al., 2010) and expressed as the total amount of MN per 1000 cells. MN frequency was determined following the examination criteria described previously (Grisolla, 2002). Briefly, criteria for MN identification in erythrocytes were as follows: diameter smaller than one-third of the main nuclei diameter, non-refractability, staining intensity similar to or lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, and an MN boundary distinguishable from the main nuclei boundary (Fenech, 2000).

Genotoxicity was tested on the basis of the presence of micronuclei and erythrocyte nuclear abnormalities (NA), following procedures described by Guilherme et al. (2008) and Lajmanovich et al. (2014). Briefly, cells having two nuclei were considered bi-nucleate (BN), while cells with a single nucleus presenting a relatively small invagination of the nuclear membrane were considered kidney shaped nuclei (K), and nuclei displaying nuclear membrane evagination were considered bud nuclei (B).

2.5.3. Integrated biomarker index

The methodology of this index involved allocating categories to the biological responses (biomarker values) based on the degree of response observed. Responses for each of the biomarkers were categorized by comparing data to the control group (NC in MHW). Data within the 95% confidence interval was regarded as belonging to “category zero”. Data outside this ± 1 SD mean response interval was assigned to “category 1”. Finally, data not included in either of the above intervals was included in “category 2”. These categories were also employed to analyze the incidence of affected individuals for each experimental condition. Thus, fish presenting 33% or more of these parameters in category 1 were classified as being “affected”, while fish presenting 33% or more of these parameters in category 2 were considered “highly affected”. The biomarker index for each experimental condition was therefore established on the basis of these values expressed as a % of affected or highly affected individuals (Amado et al., 2011).

In calculating this index, biomarkers analyzed were CAT, SOD, GST and GSH for section M and MN and NA; the calculation was performed individually on each specimen subject to each of the treatments (Rg, RgCd and Cd).

2.6. Statistical analyses

Data on biomarkers are reported as mean ± SEM. Data normality and homoscedasticity were controlled using Kolmogorov-Smirnov and Bartlett testing, respectively. One-way analysis of variance (ANOVA) was performed followed by multiple-comparison testing (Tukey). Kruskal-Wallis testing was used for cases not fulfilling the required conditions (Zar, 2010). Significance level was set at p < 0.05.

3. Results

3.1. Chemical profile of the river water sample

Results of the analyses are listed in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.7 ± 0.1</td>
<td>8.5-8.9</td>
</tr>
<tr>
<td>Chloride</td>
<td>7.7 ± 0.5</td>
<td>6.5-9.3</td>
</tr>
<tr>
<td>Nitrate</td>
<td>327 ± 45</td>
<td>275-396</td>
</tr>
<tr>
<td>DOC</td>
<td>65 ± 5</td>
<td>50-80</td>
</tr>
<tr>
<td>BOD₅</td>
<td>7 ± 0.5</td>
<td>5-9</td>
</tr>
<tr>
<td>TDS</td>
<td>80 ± 5</td>
<td>70-90</td>
</tr>
<tr>
<td>Conductivity</td>
<td>800 ± 80</td>
<td>700-950</td>
</tr>
<tr>
<td>Temperature</td>
<td>22 ± 1</td>
<td>21-23</td>
</tr>
</tbody>
</table>

Table 1. Biochemical biomarkers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (A)</td>
<td>0.7 ± 0.1</td>
<td>0.5-1.0</td>
</tr>
<tr>
<td>SOD (A)</td>
<td>0.7 ± 0.1</td>
<td>0.5-1.0</td>
</tr>
<tr>
<td>GST (M)</td>
<td>0.4 ± 0.1</td>
<td>0.3-0.5</td>
</tr>
<tr>
<td>GSH (M)</td>
<td>1.0 ± 0.1</td>
<td>0.8-1.2</td>
</tr>
</tbody>
</table>

Table 2. Integrated biomarker index

Mortality recorded in the Cd group was three individuals (15%) and in the CP group two individuals (10%). No mortality was recorded in the other treatment groups.

The C. decemmaculatus CF and results of enzyme activities, as well as GSH concentrations in A and M fraction are listed in Table 2.

CF, Pr (M), SOD (M) and CAT (M) remained stable at values similar to controls for all the treatments.

GST (M) activity decreased significantly in animals exposed to the Reconquista river (Rg) sample.

GSH (M) content was significantly lower in all treatments compared to control (NC). This reduction was more strongly marked in fish exposed to river water.

Pr (A) content decreased significantly in Rg and Cd treatments. GSH (A) content likewise decreased significantly as compared to the control group for RgCd and Cd treatments, though not for Rg.

GSH content reduction was more significant in the body.
Table 1
Physicochemical parameters and WQIs for Reconquista river water. Data are shown as mean ± SEM. WQIa: Water Quality Index (Berón, 1984); WQIb: Water Quality Index (Lacoste and Collarius, 1995).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>River water</th>
<th>Argentine guidelines (mg/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>UpH</td>
<td>8.62 ± 0.1</td>
<td>6.5–10</td>
</tr>
<tr>
<td>Conductivity</td>
<td>μS cm⁻¹</td>
<td>837 ± 5</td>
<td></td>
</tr>
<tr>
<td>Turbidity</td>
<td>UNF</td>
<td>294 ± 5</td>
<td></td>
</tr>
<tr>
<td>Alkalinity</td>
<td>mg CaCO₃/L</td>
<td>70 ± 5</td>
<td></td>
</tr>
<tr>
<td>Chlorides</td>
<td>mg Cl⁻/L</td>
<td>49.6 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>DO</td>
<td>mg O₂/L</td>
<td>2.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Ammonium</td>
<td>mg N-NH₄⁺/L</td>
<td>0.0586 ± 0.0063</td>
<td>0.05–0.47</td>
</tr>
<tr>
<td>Nitrates</td>
<td>mg N-NO₂⁻/L</td>
<td>0.017 ± 0.003</td>
<td>≤ 0.06</td>
</tr>
<tr>
<td>SRP</td>
<td>mg P-P₂O₅³⁻/L</td>
<td>0.76 ± 0.01</td>
<td>≤ 1.0³</td>
</tr>
<tr>
<td>BOD₅</td>
<td>mg O₂/L</td>
<td>7.7 ± 0.5</td>
<td>≤ 50</td>
</tr>
<tr>
<td>COD</td>
<td>mg O₂/L</td>
<td>42 ± 1</td>
<td>≤ 250</td>
</tr>
<tr>
<td>COD/BOD₅</td>
<td></td>
<td>5.45</td>
<td></td>
</tr>
<tr>
<td>WQIa</td>
<td></td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>WQIb</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Heavy metal</td>
<td>mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>&lt;</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>Pb</td>
<td></td>
<td>0.0056</td>
<td>0.001</td>
</tr>
<tr>
<td>Cd</td>
<td>&lt;</td>
<td>0.0005</td>
<td>0.00000–0.0004</td>
</tr>
<tr>
<td>Cu</td>
<td></td>
<td>2087</td>
<td>0.002–0.004</td>
</tr>
<tr>
<td>Zn</td>
<td></td>
<td>0.019</td>
<td>≤ 0.030</td>
</tr>
<tr>
<td>As</td>
<td></td>
<td>0.033</td>
<td>≤ 0.015</td>
</tr>
</tbody>
</table>

* Argentine Surface Water Guidelines for protection of aquatic life or maximum allowable content in effluents disposed in a water body.

** Total phosphorus.

midsection (as compared to the control group) than that obtained in section A.

3.3. Genetic biomarker

Though an increase in MN was recorded for all the treatments applied, significant differences in comparison with the negative control group (NC) were only found in animals exposed to Cd and to cyclophosphamide (CP), positive genotoxicity control (p = 0.02).

Total NA (BN + B + K) showed significant differences in all the treatments applied as compared to the negative control group (p < 0.001).

3.4. Integrated biomarker index

Integration of biomarkers assessed revealed that 62% of animals exposed to river samples suffered the most significant alterations (i.e. were highly affected) (Fig. 3). Among individuals exposed to Cadmium 56% were highly affected and for animals exposed to RgCd the percentage of highly affected was 55%.

Table 2 indicated that GST and GSH biomarkers from fish exposed to Reconquista river water (Rg) varied significantly from control values, as did those for NA (Table 3).

Biomarker categorization using the Integral Biomarker Index showed that fish parameters were affected and had differential responses (categories 1 or 2) depending on the medium they were exposed to. Considerable differences were registered in fish exposed to different water samples with a significant deterioration of the parameters being analyzed. The most strongly affected parameters in category 2 were GSH and NA, whereas in category 1 the most highly affected was MN (Table 4). When considering the integral response of the fish (biomarker index), 80% of individuals were classified as highly affected (Fig. 3).

4. Discussion

Several water bodies, e.g. rivers and lakes, are receptors of wastes generated for industry, agriculture, and urban water treatment plants, containing a mix of many pollutants and known...
toxicants, whose toxic biological effects on the biota are hardly any detectable. In Argentina, the Reconquista river is the second most polluted river (Salibián, 2006). The toxicological quality of its course has been previously investigated by several researchers (de la Torre et al., 2005, 2007; Salibián, 2006; Ossana et al., 2013; Ricacci et al., 2013).

Chloride, phosphate and inorganic nitrogen compound concentrations are not high, but heavy metal concentrations were found to be higher than the local legislation guidelines for protection of aquatic life, in particular copper, which exceeded this limit by several orders of magnitude (Law 24,051). This is reflected in the WQb Index and denotes high contamination of industrial origin.

In the upper Reconquista river basin the predominant processes regulating or deteriorating the physical and chemical characteristics of the water are subsurface runoff and specific industrial and municipal effluent discharges. The La Choza stream provides the greatest contaminant load into the Roggero dam. This is mainly a consequence of discharge from point wastewater unloading such as that generated by a small industrial park located near the mouth of the stream. At this site there is, among others, an agrochemical industry and poultry slaughter, which continuously discharges effluents into the stream. Studies at this site showed high biochemical oxygen demand (BOD5) values and suspended solid levels as a consequence of organic pollution (Basílico, 2014; Ricacci et al., 2013).

In this study, the biochemical biomarkers analyzed revealed a lower GST activity and GSH levels in animals exposed to river water samples when compared with control group.

Redox-active metals such as iron, copper and chromium undergo redox cycling, whereas redox-inactive metals such as lead, cadmium, mercury and others deplete cell major antioxidants, particularly thiol-containing antioxidants and enzymes. Either redox-active or redox-inactive metals may cause an increase in ROS production. GSH is also a cofactor for various enzymes like GST and itself decreases oxidative stress. GSH is abundant in the liver, and is thought to be the first line of defense against Cd hepatotoxicity (Rani et al., 2014). Our results showed how GSH content decreased in NC > Cd > RgCd > Rg treatments. GSH content in the anterior section of C. decemmaculatus individuals also fell, and was registered as NC > Rg > RgCd > Cd.

Antioxidant enzymes and non-enzymatic systems are essential for the conversion of reactive oxygen species into harmless metabolites and for the protection and restoration of normal metabolism and cell function. It is not a general rule that an increase in a xenobiotic concentration could induce enzyme activity (Bonifacio et al., 2018). In our case the addition of a Cd contamination pulse to the river water did not increase biomarker activity and did not affect CAT, SOD and GST activity. Bonifacio et al. (2016) found that in Ctenosynapsis decemmaculatus exposed to Clorox® and Roundup Max® and a mixture thereof, liver CAT activity showed differing results, but the combined effect of the mixture was not worse than the effect of the chemicals when applied individually.

Scarcia et al. (2014) studied the impact in juveniles of Cyprinus carpio and Pimelodella laticeps in situ exposed to two contaminated sites in the Lujan river for 14 days and they found a significant increase in SOD and GST though CAT remained unchanged.

Dabas et al. (2012) studied the effects of the exposition to Cd sublethal concentrations during 96 h in Channa punctatus. All enzymes activities, except CAT (in kidney and gills), as well as LPO levels increased significantly compared to control values. Results indicated that the increase in LPO level and the fluctuation in fish antioxidant defense system could be due to a Cd-induced increase in the production of reactive oxygen species (ROS). Other authors exposed Rutilus rutilus caspicus to CdCl₂ and Pb sublethal concentrations for 96 h. Both heavy metals were highly toxic in R. rutilus caspicus muscle, with lead showing higher toxicity than Cd in terms of nutritional parameters and antioxidant enzyme activities (Raesi et al., 2015).

A variety of responses were found depending on the species, tissues and toxics analyzed. River water is a complex matrix and the addition of a cadmium pulse does not necessarily lead to increased oxidative stress for individuals exposed to this medium.

There was a significant increase in MN frequency in animals exposed to Cd and in the positive genotoxicity control tests (CP). These results confirm previous findings showing the genotoxic effect of Cd, which damages fish DNA, presumably by inhibiting repair mechanisms (Rani et al., 2014). This study also revealed a very significant increase in NA frequency which occurred in all the treatments applied. NA frequency observed in C. decemmaculatus erythrocytes was, from highest to lowest: RgCd > Cd > Rg > CP > NC. This would indicate that the Cd contamination pulse is a potential inductor of genomic instability in C. decemmaculatus erythrocytes, an effect that increased in the river water sample. Previously, Gürer and Gökal Muranli (2011) studied MN and NA frequency in Gambusia affinis following exposure during 1 and 2 weeks to cooper (Cu) and Cd and found that combined exposure to these two metals increased NA frequency in a larger proportion than when individuals were exposed to each of these metals separately. These results are similar to our own given that the highest frequency was noted in RgCd treatment which involved a combination of Cu and Cd.

The Integral Biomarker Index shows that fish exposed to different treatments were highly affected, with GSH, AN and MN being the biomarkers displaying the greatest effects, in addition to CAT among fish exposed to river water (Table 4).

This integrated index indicates a degree of deterioration and reveals differences between treatments that are difficult to quantify separately. Some biomarkers did not seem to be affected, (CAT) yet when analyzed using the index it is clear that a large number of exposed individuals were found to have been affected.

### Table 4

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Rg</th>
<th>RgCd</th>
<th>Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (M)</td>
<td>15</td>
<td>60</td>
<td>10.5</td>
</tr>
<tr>
<td>GST (M)</td>
<td>37</td>
<td>31.5</td>
<td>37</td>
</tr>
<tr>
<td>GSH (M)</td>
<td>0</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>SOD (M)</td>
<td>37.5</td>
<td>12.5</td>
<td>33</td>
</tr>
<tr>
<td>MN</td>
<td>90</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>NA</td>
<td>25</td>
<td>70</td>
<td>5</td>
</tr>
</tbody>
</table>

5. Conclusion

The results submitted in this paper reveal significant water quality deterioration at the headwaters of the Reconquista river, with a large concentration of heavy metals. This would lead to a stressful situation for the river biota. This was noted in both the enzymatic and non-enzymatic biomarkers assessed, in particular for GST and GSH which showed the greatest variation. In the genotoxicity field, genomic instability was found in the erythrocyte DNA; while MN frequency increased in positive controls (Cd and PC), NA increased significantly for all the treatments applied, with the highest level found in RgCd. However, the multiple biological responses from different biomarkers is hard to correlate...
with a specific toxic and water quality index, and in this sense the application of an integrated biomarker index is promising. This index showed that water in the early sections of the river Re- conquista induces enzymatic, non-enzymatic and genetic changes in exposed animals, which are categorized as being highly affected (Fig. 3). In fish exposed to Rg, RgCd and Cd, over 70% were affected or highly affected. All these changes were observed following acute exposure for 96 h where no compensatory responses are expected to occur.

An integrated analysis is required using a broad variety of biomarkers with the purpose of obtaining further information as to the stress undergone by the organisms, and likewise using reference toxics.

This study made use of a native species living in different parts of the Reconquista river that exhibiting variable degrees of pollution and our results showed that C. decemmaculatus would be being affected. It is interesting the promptness of their response which has led to their being used as a sentinel species in brief toxicity bioassays.

Acknowledgments

This work was supported by grants from the National University of Luján CDD-CB 540/14 (Basic Sciences Department) and the ANPCyT (PICT-2012). We thank the “Los Robles” Municipal Reserve for their collaboration in the field surveys, Martina Marta for her collaboration in water chemical determination and Luis Tripoli and Jesica Benitez for support on the fish culture maintenance. The manuscript was improved by the helpful comments from anonymous referees.

References


Bird & Girard, 1853) Turkis. J.


