Research Article

Responses of biomarkers of a standardized (*Cyprinus carpio*) and a native (*Pimelodella laticeps*) fish species after *in situ* exposure in a periurban zone of Luján river (Argentina)

1. Paola Scarcia[1,2],
2. Gabriela Calamante[3] and
3. Fernando de la Torre[1,2,*]

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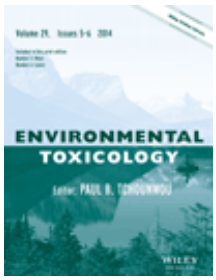

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Author Information

Responses of Biomarkers of a Standardized (*Cyprinus carpio*) and a Native (*Pimelodella laticeps*) Fish Species After *In Situ* Exposure in a Periurban Zone of Luján River (Argentina)

Paola Scarcia,[1,2] Gabriela Calamante,[3] Fernando de la Torre[1,2]

[1]Programa de Ecofisiología Aplicada (PRODEA-INEDES), Departamento de Ciencias Básicas, Universidad Nacional de Luján, Casilla de Correo 221, B6700ZB, Luján, Argentina

[2]Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires, Argentina

[3]Instituto de Biotecnología, CICVyA-INTA, Castelar, Argentina

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ABSTRACT: The Luján River basin, which is located in the northwest area of the province of Buenos Aires, Argentina, receives different anthropogenic inputs before reaching the Río de la Plata estuary. The aim of this study was to assess the adverse impact of the river in the middle part of the basin. To this end, an *in situ* cage assay was conducted in two sites of the river (S1 and S2) near Luján city, and the responses of hepatic biomarkers of both a standardized (*Cyprinus carpio*) and a native (*Pimelodella laticeps*) species were evaluated. The biomarkers studied were the condition factor and liver somatic indices (LSI), the enzymatic activities of catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST), lipid peroxidation levels (thiobarbituric acid reactive substances, TBARS) and the induction of hepatic cytochrome P450 1A (CYP1A) and vitellogenin (Vtg) proteins. After 14 days, LSI and GST activity increased, and TBARS levels decreased in both species exposed at S1 and S2. In addition, exposure at both sites promoted an increase in SOD activity and CYP1A induction in *C. carpio*, while Vtg expression was observed only at S1. A shorter exposure period (7 days) caused an initial response only at S2 mediated only by CAT in *P. laticeps*. Finally, our results demonstrate that a 14-day period of *in situ* exposure in Luján River could lead to antioxidant and biotransformation processes in *C. carpio* and to phase II biotransformation responses in *P. laticeps*. © 2012 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2012.

Keywords: biochemical and molecular biomarkers; *Cyprinus carpio*; field caging assay; periurban Luján River; native fish species

INTRODUCTION

The excessive development of human activities, which leads to increased levels of pollutants, can affect the biological integrity of aquatic environments. In the northwest of the Province of Buenos Aires (Argentina), the adverse impact of anthropogenic pollution in numerous water bodies has been reported by several authors (Giorgi et al., 1999; de la Torre et al., 2000). In particular, the Luján River

Correspondence to: F. de la Torre; e-mail: fdelatorre@mail.unlu.edu.ar

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basin covers an area of 2600 km^2 and crosses the northwest part of the province of Buenos Aires and after 128 km empties its waters into the Río de la Plata estuary (AABA, 2010). The river is divided in three stretches: upper, middle, and lower reaches (O'Farrell et al., 2002). The middle reach (30 km), which is the sector of the basin that has the most pronounced relief and the greatest drainage, extends from Jáuregui up to the town of Pilar, crossing Luján district (Giorgi et al., 1999). The river receives different types of contamination, with the main contributions provided by punctual urban and industrial liquid discharges that are incorporated progressively in downstream direction when the agricultural use of the land is replaced by an urban-industrial one. The city of Luján is an example of this type of impact, where the municipal Wastewater Treatment Plant (WWTP) pours a daily flow of 11,000 m^3 into the river (Sánchez Caro, 2010). In addition, the middle reach of the river receives the adverse impact of many industries such as breweries, dairy, tannery, food and textile industries, which discharge their waste either into the sewage system or directly into surface waters (Di Marzio et al., 2005; Sánchez Caro, 2010).

Anthropogenic pollutants discharged into the rivers, either by punctual and diffuse inputs, can be incorporated by aquatic organisms through different routes: directly from water, via uptake of suspended particles, or by the consumption of lower trophic level organisms. Once in an organism, persistent compounds may bioaccumulate or undergo biotransformation via phases I and II so as to be excreted. These processes occur mainly in the liver and to a lesser extent in other tissues. In fish, the cytochrome P450 1A (CYP1A) is the isoform responsible for biotransformation of many hydrocarbons such as policyclic aromatic hydrocarbons (PAHs), some congeners of polychlorinated biphenyles (PCBs), and dioxins (van der Oost et al., 2003). The quantification of CYP1A protein, mainly by immunological methods, has proven to be a useful tool for monitoring aquatic environments polluted with PAHs (Fent, 2004; Bozcaarmutlu et al., 2009). On this basis, Western blot analysis using specific polyclonal antibodies against CYP1A was performed in this study. Phase II involves the conjugation of xenobiotic compounds or their metabolites with endogenous ligands. In this context, glutathione-S-transferase (GST) provides cellular protection against the toxic effects of a variety of endogenous and environmental chemical compounds such as PAHs, endosulphan, and microcystins (van der Oost et al., 2003; Schlenk et al., 2008). On the other hand, reactive oxygen species (ROS) are generated during the metabolism of numerous pollutants present in the aquatic environment, leading to oxidative stress in organisms. Fish, like mammals, possess a well-developed antioxidant defense system to neutralize toxic effects of ROS (Livingstone, 2001; Ahmad et al., 2004). This system includes antioxidant enzymes such the superoxide dismutase (SOD) and catalase (CAT). Many biochemical and physiological effects (i.e., lipid peroxidation, LPO) have been associated with an increment in ROS and may serve as good indicators of oxidative stress. LPO in fish, measured as thiobarbituric acid reactive substances (TBARS), has often been used as an effective biomarker of toxic pollutants such as Cu, Fe, Cd, PAHs, and PCBs in a number of studies (Livingstone, 2001; Oakes and van der Kraak, 2003; Almroth et al., 2005; Song et al., 2006). It has to be mentioned that the industrial and urban discharges in peri-urban rivers are potential sources of substances that could lead to endocrine disruption in aquatic organisms. Among these, some metabolites of polyetoxylated alkylphenols (i.e., nonylphenol, NP) could mimic endogenous estrogen and their estrogenic action can be evaluated in male and juvenile fish by the presence of vitellogenine protein (Vtg) in plasma (Mitchelmore and Rice, 2006).

The ecotoxicological assessment of aquatic contamination can be performed using different methodologies. In aquatic biomonitoring, *in situ* approaches have been shown to be more appropriate than chemical analyses, laboratory tests, and resident biota studies. Biomarkers have been proposed as integrative and sensitive tools for the ''early detection'' of biological effects and have, therefore, been incorporated in various environmental monitoring programs (van der Oost et al., 2003). The responses of a ''battery'' of physiological, biochemical, and molecular biomarkers have been frequently used to detect or diagnose sublethal effects in fish (van der Oost et al., 2003; Oikari, 2006; Monserrat et al., 2007). Moreover, the simultaneous use of several biomarkers is important to minimize possible misinterpretations, mainly in cases of complex situations of contamination (Linde-Arias et al., 2008). In this context, *in situ* cage assays conducted with carps, in which biomarker responses are evaluated, have shown the usefulness of this technique under different situations when reporting the quality of aquatic environments (van der Oost et al., 1998; de la Torre et al., 2000; Smolders et al., 2004; Bervoets et al., 2009).

Fish have been widely used as test organisms. In this study, we used the standardized species *Cyprinus carpio* and the native species *P. laticeps*. *C. carpio*, which in Argentina is found as an exotic species in many water bodies, was chosen because of its suitability in toxicity tests and handling conditions. *P. laticeps* is a native bottom dweller species, and its presence has also been reported in many rivers and ponds of Buenos Aires province as well as in the Luján River basin (Liotta, 2006). This species was chosen because it is also suitable for laboratory and field bioassays. The aim of this study was to assess the adverse impact of Luján River in areas close to the city of Luján, using an *in situ* biological approach by means of hepatic biomarker responses of a standardized species (*C. carpio*). In addition, using the same experimental methodology, we evaluated biomarkers in a representative native species (*P. laticeps*) at two sampling moments and explored the potential of early responses of the parameters.

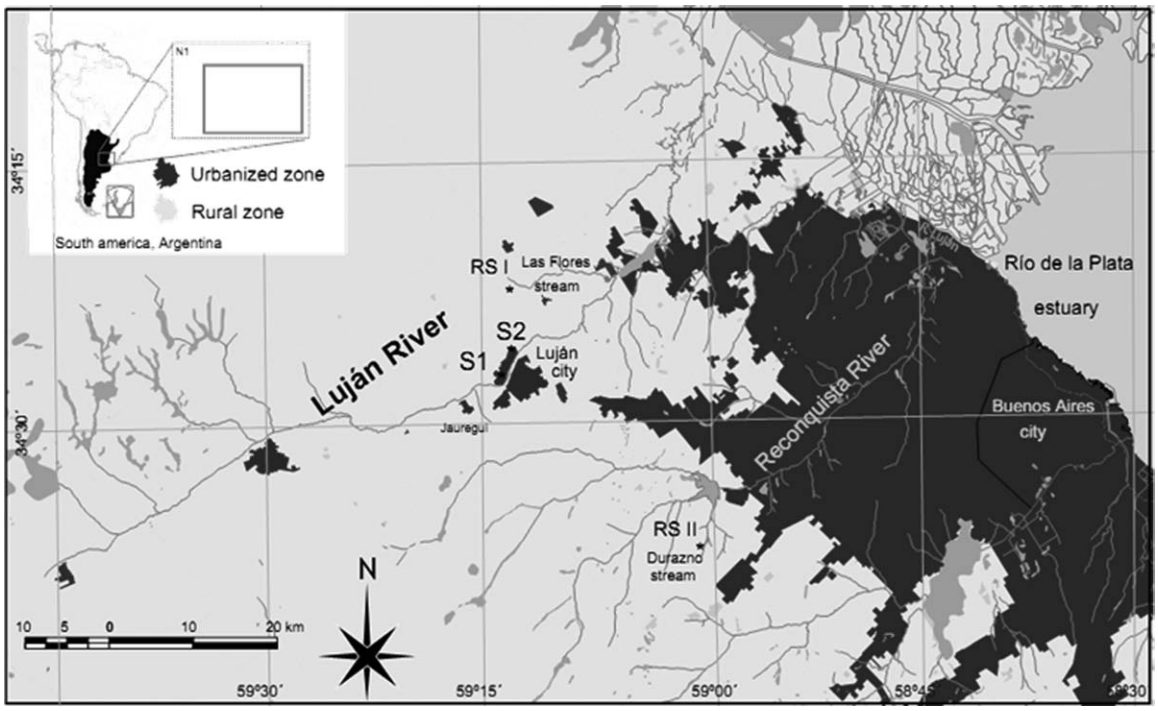

Fig. 1. Map of Luján and Reconquista River basins. S1 and S2 indicate the experimental sites located upstream and downstream of the municipal waste water treatment plant of Luján, respectively; RSI and RSII correspond to reference sites.

MATERIALS AND METHODS

Test Organisms

To minimize eventual sex-related variability in biomarker responses, only sexually immature fish were used. Juveniles of *C. carpio* (total $n = 33$) with a body weight (b.w.) of 3.7 ± 0.1 g and a total length of 6.3 ± 0.1 cm were obtained from a local commercial hatchery and *P. laticeps* (total $n = 50$) with a b.w. of 4.8 ± 0.1 g and a total length of 8.8 ± 0.1 cm (mean ± SEM) were collected from streams with low anthropogenic impact. Fish remained 2 weeks under laboratory standardized conditions in 20-L aquaria with a continuous flow (25 mL/min) of nonchlorinated tap water free of contaminants, keeping fish with a body weight density of 1.8 g/L. The temperature was kept at 24 ± 1°C and the photoperiod was 12 h light/12 h dark. Fish were daily fed with commercial fish food (1% of body weight) having the following composition: protein 47%, fiber 2%, moist 10%, and ashes 13%.

Study Area and *In Situ* Tests

The study was conducted in two sites (S1 and S2) of Luján River placed in the district of Luján. S1 was located upstream the municipal WWTP of Luján city, where the nearest source of contamination is provided principally by textile industry discharges poured directly into Gutiérrez stream (Giorgi and Malacalza, 2002). S2 was located downstream the WWTP, where the toxicity of river water is increased by the impact of the effluents. Two reference sites were chosen: Las Flores stream (RSI), a middle reach tributary of Luján River, and Durazno stream (RSII), located in the upper part of the Reconquista River (Fig. 1). The upper part of Las Flores stream has no anthropogenic impact, and its sediment is practically free of contaminants, making it good for potential use as reference control materials for toxicity assessment surveys in the region (Ronco et al., 2008). At the same time, the headwaters of Durazno stream was proposed as a reference site because of its water quality (low nutrients and high oxygen levels) and low population (Arreghini et al., 2007).

C. carpio In Situ Test

Juveniles of *C. carpio* were simultaneously exposed at S1, S2, and RSII for 14 days during early spring of 2007 in perforated polypropylene cages ($n = 11$, per site; 2.0 g b.w./L). The cages were 50 cm × 25 cm × 16 cm, with a mesh size of 8 mm in diameter.

Two additional groups of fish (total $n = 20$) remained under the same experimental conditions at RSI, to obtain additional data to be considered together with RSII as field reference values (RV) of each biomarker.

P. laticeps In Situ Test

Similarly, juveniles of *P. laticeps* ($n = 16$, per site; 2.0 g b.w./L) were exposed at the same experimental sites

S1 and S2 under the same conditions during autumn 2008, whereas the reference group remained at RSI. In this case, after 7 days of exposure, a group of eight fishes of each site were sampled and the remaining fish remained exposed for 14 days.

Water Physicochemical Parameters

The water physicochemical parameters were measured in surface water samples taken from both experimental and both reference sites. Samples were collected in duplicate and transported to the laboratory at 4°C. The analysis included soluble reactive phosphorus, ammonium, and nitrites; all measurements were made twice following standard procedures (APHA, 2005). In addition, temperature, pH, dissolved oxygen, and conductivity were measured *in situ* using Hanna Instruments electrodes (HI 98240; HI 9142; HI 933301).

Preparation of Biological Samples

After the experimental period, fish were immediately transferred to the laboratory in aerated containers and anesthetized with MS-222 (3-aminobenzoic acid ethyl ester methane sulfonate salt. Sigma-Aldrich, St. Louis, MO). Body weight and length were recorded. Fish were bled from the caudal vein using a heparin-coated syringe, and 10 μL of protease inhibitor cocktail (Sigma-Aldrich) was added to all samples. Fish were then sacrificed by an incision behind the operculum, and the liver excised and weighed. After these procedures, blood samples were centrifuged at 1000 $\times$ *g* for 15 min, the pellet discarded, and the plasma used to evaluate the presence of Vtg. Aliquots of liver and plasma samples were kept at -80°C until processed. Aliquots of liver were individually homogenized on ice until total disintegration with buffer pH 7.4 (0.1 M NaH_2PO_4; 0.15 M KCl; 1 mM EDTA; 1 mM DTT; 10% v/v glycerol) according to Nilsen et al. (1998). The sample homogenates were centrifuged at 10,000 $\times$ *g* for 15 min at 4°C and the obtained supernatant fraction (SF) was reserved for evaluation of enzyme activities, protein content, and presence of CYP1A. Another aliquot of liver from each individual was homogenized 10% w/v in 1.15% KCl with 35 μM butylated hydroxytoluene, this homogenates were reserved for determination of LPO (Oakes and van der Kraak, 2003).

Biomarkers: Physiological Indices and Biochemical and Molecular Parameters

The following physiological indices were evaluated: the condition factor (CF), which was calculated as (b.w. (g)/total length^3 (cm^3)) $\times$ 100 (Bagenal and Tesch, 1978), and the liver somatic index (LSI), which was calculated as (liver weight (g)/b.w. (g)) $\times$ 100 (Sloof et al., 1983).

CAT activity (EC 1.11.1.6) was determined according to Beers and Sizer (1952), evaluating the breakdown of H_2O_2 over time, at a wavelength of 240 nm. The mixture reaction contained the aliquots of SF incubated in sodium phosphate buffer pH 7.2 (50 mM) containing H_2O_2 20 mM. The enzyme activity was expressed as μmoles of H_2O_2 consumed/min/mg of total protein.

SOD activity (EC 1.15.1.1) was evaluated following an indirect method involving the inhibition of cytochrome *c* reduction by the competition with SOD for the superoxide anion radical ($O_2^{\cdot -}$) formed by the xanthine/xanthine oxidase system (McCord and Fridovich, 1969). The reaction mixture had 50 mM potassium phosphate buffer pH 7.8, 100 μM EDTA solution, 10 μM cytochrome *c*, and xanthine oxidase (50 units). Changes in the absorbance were recorded at 550 nm. The activity was expressed as units of SOD/mg protein, where 1 SOD unit (U) is defined as the enzyme quantity that causes 50% of inhibition of reduction of cytochrome *c* per minute.

GST activity (EC 2.5.1.18) was evaluated using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, which can be conjugated with the different isoforms of GST in the presence of reduced glutathione (GSH); the presence of the formed conjugate was evaluated by spectrophotometry at 340 nm. The assay conditions were: sodium phosphate buffer pH 6.5 (0.1 M), 10 mM GSH, and CDNB 20 mM (Habig et al., 1974). Enzyme activity was expressed as GS-CDNB μmoles formed per minute and mg of total protein.

All enzyme activities were evaluated at 25°C and were referred to the total protein content. In this context, protein content in the SF was determined using serum bovine albumin as standard (Lowry et al., 1951).

LPO was measured as malondialdehyde by the thiobarbituric acid reaction, under acidity and heat conditions. The chromogen formed was measured by fluorometry according to Ohkawa et al. (1979) and Oakes and van der Kraak (2003). The 10% homogenate was added to a mixture composed of 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid (pH 3.5), 0.8% thiobarbituric acid, and butylated hydroxytoluene 35 μM solutions. The tubes were incubated at 95°C for 1 h. After cooling, Milli-Q water and *n*-butanol (99.4%) were added, and the mixture mixed by inversion. Finally, the mixture was centrifuged at 2000 $\times$ *g* for 10 min at 15°C to separate the organic layer, which was measured by fluorometry at an excitation wavelength of 515 nm (slit width 10 nm) and an emission wavelength of 553 nm (slit width 5 nm). The malondialdehyde concentration was expressed as nmol TBARS/g of tissue; tetramethoxypropane was used as an external standard. A positive control of LPO was performed with Fe^{3+} because of its ability to increase ROS production ($^{\cdot}OH$, H_2O_2) via Fenton and Haber-Weiss-like reactions. *In vitro* assays were performed with Fe^{3+}, and liver homogenates from *C. carpio* incubated for 2 h with increasing concentrations of Fe^{3+} (0, 5, 10, and 50 μM).

TABLE I. Surface water physicochemical parameters of Luján River and reference streams

	Sites of Luján River		Reference Sites		
Parameters	S1	S2	RSI	RSII	MPQ
Temperature (°C)	16 (14–28)	16 (13–21)	14 (13–24)	15 (10–27)	
pH (pH units)	7.8 (7.5–8.4)	7.7 (4.6–8.4)	7.6 (6.8–8.1)	8.5 (8.3–8.6)	7.5 − 8.5b
Conductivity (mS/cm)	2.66 (2.4–2.75)	2.43 (2.23–2.53)	0.85 (0.67–0.95)	0.85 (0.66–1.01)	
Dissolved oxygen (mgO_2/L)	5.7 (4.6–8.1)	4.5 (3.4–7.9)	6.2 (4.2–8.1)	8.5 (6.9–9.4)	> 4b
Soluble reactive phosphorus (mg/L)	0.98 (0.24–1.60)	1.04 (0.54–1.83)	0.43 (0.18–0.61)	0.14 (0.06–0.34)	
Ammonium (mg N-NH_4^+/L)	0.93 (0.16–2.81)	2.71 (0.52–8.00)	0.03 (0.00–0.06)	0.02 (0.00–0.07)	1.13a
Nitrites (mg N-NO_2^-/L)	0.73 (0.37–1.63)	0.81 (0.03–1.36)	0.04 (0.02–0.06)	0.03 (0.01–0.06)	0.06a

Note: Values are expressed as median and (minimum–maximum) values. MPQ: maximum permitted quantities according to the Law No. 24051 (a) for protection of freshwater life and (b) according to Berón (1984).

All measurements were conducted at least twice using analytic grade reagents.

The presence of CYP1A or Vtg proteins was assessed by Western blot. Briefly, samples with equal amounts of protein (100 or 40 μg, respectively) were loaded onto polyacrylamide gel (sodium dodecyl sulfate 12% or 8% polyacrylamide gel electrophoresis, respectively) and, after electrophoresis, proteins were transferred onto nitrocellulose membrane (Ausubel et al., 2005). Transference to nitrocellulose membrane was performed for 1 h at 60 V (for CYP1A) or on ice at 100 V for 90 min (for Vtg) in 50 mM Tris pH 7.5, 196 mM glycine, 20% methanol. The nitrocellulose membrane was incubated overnight in blocking buffer (phosphate-buffered saline containing 5% w/v nonfat milk powder and 0.1% Tween 20) at 4°C. The membrane was incubated for 1.5 h at room temperature (20–25°C) with anti-CYP1Ar mouse serum (de la Torre et al., 2003) diluted in blocking buffer (1:2000) or overnight at 4°C with mouse anti-carp Vtg (monoclonal antibody, ND-2D3, Biosense Laboratories, Bergen, Norway) diluted in blocking buffer (1:1000). After three washes with TBS-Tween, the membrane was incubated for 1.5 h with an alkaline phosphatase-conjugated anti-mouse-specific IgG (Sigma-Aldrich) diluted in blocking buffer (1:20,000). Bound antibodies were visualized by 5-bromo-4-chloro-3-indolyl-phosphate (0.165 μg/mL) and nitro blue tetrazolium (330 μg/mL) substrate precipitation (Promega, Madison, WI).

SF samples of liver from β-naphthoflavone (BNF; Sigma-Aldrich)-treated and untreated fish of both species were included as a standard reference in the CYP1A detection assay to control the stability of the measurement conditions throughout the sample set. These samples (CYP1A induction) were generated by intraperitoneal injection of 50 mg/kg BNF in corn oil (positive control). On the other hand, a positive control of Vtg induction was performed by a single intraperitoneal injection with 10 mg/kg of 17β-estradiol in corn oil (E2; Sigma-Aldrich); in the case of *C. carpio* adult males were also injected with the same dose of E2. NP (Sigma-Aldrich), another compound that could be present in the environment and is known because of its estrogenic action, was injected in both species (100 mg/kg NP in corn oil). Fish were kept in the standardized conditions described in Test Organisms section, and livers (CYP1A induction) were sampled 48 h after exposure and plasma samples (Vtg induction) were obtained after 72 h exposure and then processed as described previously.

Statistical Analysis

Data distributions were tested for normality and variance homogeneity with Kolmogorov–Smirnov and Levene's tests, respectively. Data that did not fulfill the parametric requirements were adjusted, transforming them either by means of the logarithm or the reciprocal of the variables. Statistical differences ($p < 0.05$) between groups were determined using analysis of variance followed by Tukey's test (Zar, 1996).

RESULTS

No mortality was recorded in either experimental group during the assay period.

Water Physicochemical Parameters

Some physicochemical characteristics of the surface water of Luján River and reference sites are given in Table I. Regarding the parameters measured in the field, similar values of temperature and pH were recorded in both assays and could be considered within the range of acceptable levels according to Berón (1984). Besides, conductivity values of both sites of Luján River (S1 and S2) were always higher than those of the reference sites (RSI and RSII). In contrast, the dissolved oxygen levels detected in the middle stretch of the river (S1 and S2) were lower than those of both reference sites, although always higher than the RV (Berón, 1984). Nutrients of river water samples were increased; in particular, ammonium and nitrite levels were higher than the maximum permitted quantities (MPQ) established by the Argentinean Law No. 24051 for protection of freshwater life. On the other hand, the values of these nutrients found in the reference sites RSI and II were within the limits allowed by the same law.

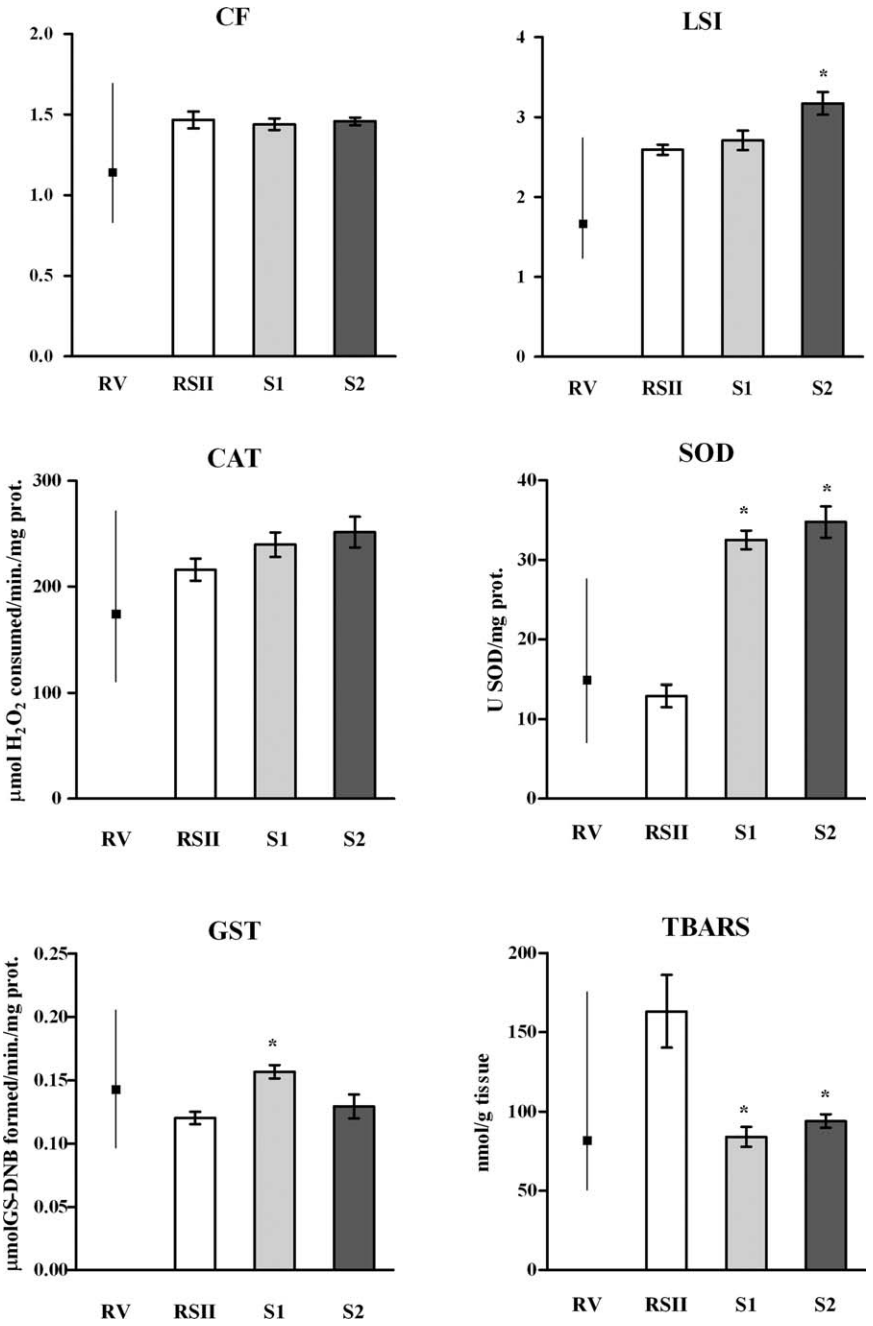

Fig. 2. Physiological and biochemical biomarkers of *C. carpio* juveniles exposed for 14 days in two sites of Luján River (S1–S2) and Durazno stream, reference site (RS II). CF: control factor; LSI: liver somatic index; CAT: catalase; SOD: superoxide dismutase; GST: glutathione-S-transferase; TBARS: thiobarbituric acid reactive substances. Values of bars indicate mean ± SEM; (n = 11). RV are expressed as median (■) and the minimum-maximum range (—) (n = 20). (*) indicate significant differences from the reference site ($p < 0.05$).

Biomarkers: Physiological Indices and Biochemical and Molecular Parameters

C. carpio In Situ Test

No differences were observed in CF of exposed river fish, whereas LSI increased by 15% in S2 and RSII (Fig. 2). With regard to the antioxidant responses, CAT denoted no changes, whereas SOD had a three-fold increase in fish exposed to S1 and S2 (Fig. 2); at the same time, TBARS levels decreased in both sites. In contrast, *in vitro* TBARS assays with Fe^{3+} promoted an increase in the level of malondialdehyde, depending on the concentration tested

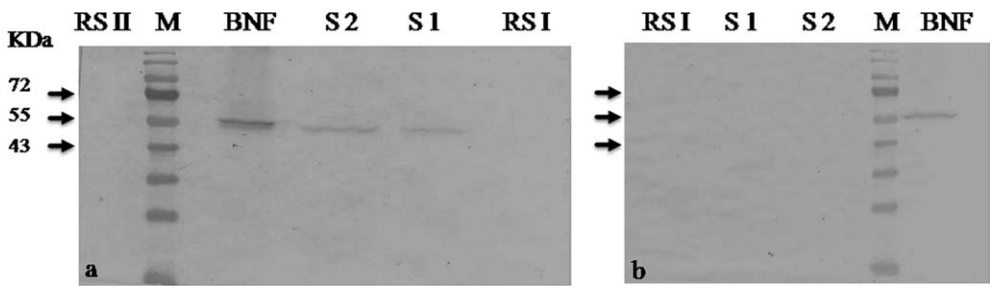

Fig. 3. Western blot analysis of CYP 1A of *C. carpio* (a) and *P. laticeps* (b). SF samples from fish placed at two experimental sites of Lujan river (S1 and S2) and reference sites (RSI and RSII, Las Flores and Durazno streams, respectively); BNF: PMS samples from fish 48 h after injection with BNF (50 mg/kg); M: molecular weight marker (Page Ruler™ Prestained Protein Ladder, Fermentas).

(data not shown). These results indicate that the hepatic tissue of *C. carpio* has a high capacity of response to a known pro-oxidant agent.

In situ river exposure also promoted changes in GST levels of fish only in S1 (29%) in relation to RSII (Fig. 2). The molecular biomarkers were based on Western blot analysis of protein samples from *in situ* exposed fish. The existence of reactive bands indicates the presence of cytochrome P4501A in samples from fish *in situ* exposed to S1 and S2. The antibody anti-CYP1Ar recognized a protein band of the appropriate molecular weight range reported for fish CYP1A (55–65 kDa). As expected, a positive signal was also detected in protein extracts from BNF-injected fish, whereas no CYP1A was detected in samples from RSI and RSII [Fig. 3(a)]. The results of Western blot analysis of plasma samples of *C. carpio* revealed the presence of reactive bands of high molecular weight (130–170 kDa) in E2 samples from injected adult males and juveniles, carps inoculated with NP and fish that remained exposed in S1 [Fig. 4(a,b)]. No reactive bands were observed in samples of carp exposed in the reference sites (RSI and II) or those from the other site of Luján River (S2) [Fig. 4(c)]. The results shown in Figures 3 and 4 represent samples from one fish from each *in situ* exposed group as similar results were obtained for the rest of fish from every group (data not shown).

P. laticeps In Situ Test

A 14-day exposure period in the river caused a significant increase in both physiological indices (Fig. 5): CF increased by 8% in S1, whereas LSI increased by 41% in S1 and by 71% in S2. With regard to the biochemical biomarker responses, the same period did not promote significant differences in SOD or CAT activities, whereas GST activities were induced in both S1 (60%) and S2 (71%). Finally, TBARS levels were significantly decreased (26% in S1 and 29% in S2) in relation to RSI (Fig. 5). The Western blot analysis of CYP1A expression in *P. laticeps* showed the presence of reactive bands only in fish injected

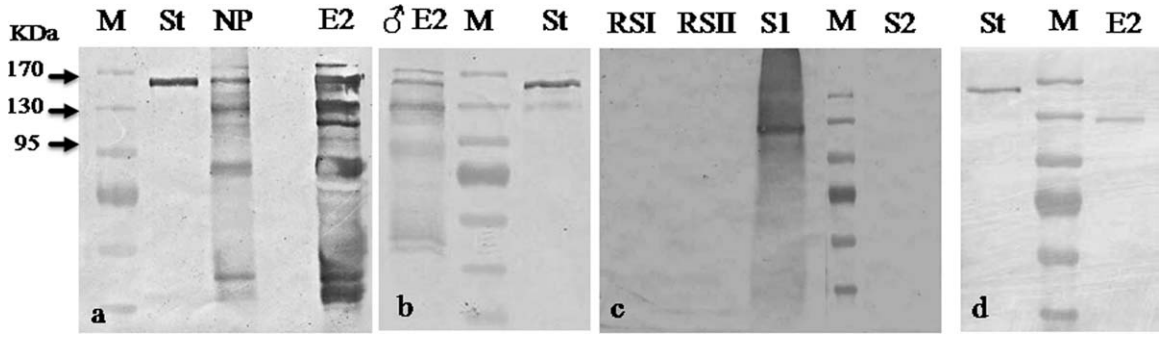

Fig. 4. Western blot analysis of Vitelogenin (Vtg) of *C. carpio* (A, B, and C) and *P. laticeps* (D). E2 and ♂E2: plasma samples from a juvenile and a male collected 72 h after injection with 17β-estradiol (10 mg/kg); NP: plasma sample from a juvenile collected 72 h after injection with NP (100 mg/kg); St: carp Vtg purified protein (Biosense Laboratories) (0.5 μg); plasma samples from two experimental sites of Lujan River (S1 and S2) or from reference sites (RSI and RSII, Las Flores and Durazno streams, respectively); M: molecular weight marker (Page Ruler™ Prestained Protein Ladder, Fermentas).

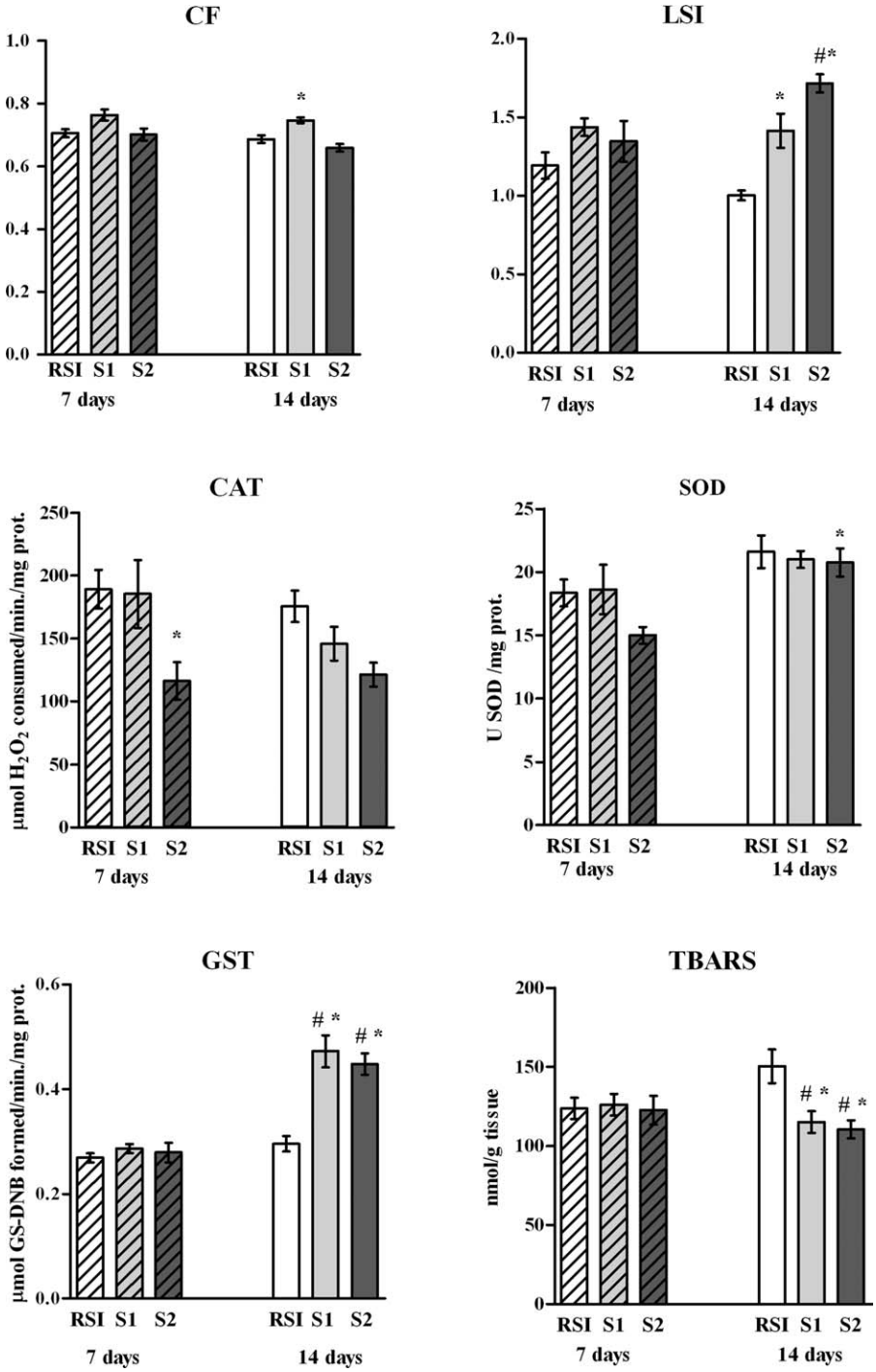

Fig. 5. Physiological and biochemical biomarkers of *P. laticeps* juveniles exposed for 7 and 14 days in two sites of Luján River (S1–S2) and Las Flores stream (RSI). CF: control factor; LSI: liver somatic index; CAT: catalase; SOD: superoxide dismutase; GST: glutathione-S-transferase; TBARS: thiobarbituric acid reactive substances. Values are expressed as mean ± SEM; ($n = 11$). (*) indicate significant differences from the reference site at the same exposure time ($p < 0.05$); (#) indicate significant differences between exposure time for the same site ($p < 0.05$).

with BNF (positive control). On the other hand, no CYP1A-specific signal was observed in exposed fish at S1, S2, and RSI [Fig. 3(b)]. Immunodetection of *P. laticeps* Vtg is represented by Figure 5(d), which shows reactive bands in samples from 17 β-estradiol-injected fish (E2). No induction of Vtg expression was observed for NP-treated

fish or *in situ* exposure in reference or experimental sites (data not shown).

When a shorter exposure period (7 days) was evaluated, no significant changes in the physiological indices were observed in fish from Luján River, when compared with the reference site (RSI) (Fig. 5). Biochemical responses showed differences in relation to RSI only in CAT activity from exposed fish at S2 (−38%) (Fig. 5).

DISCUSSION

Studies about water quality of Luján River and its tributary streams are based mainly on the assessment of nutrient/physicochemical parameters and structure of the phytoplankton community together with basic hydrological data (Giorgi et al., 1999; O'Farrell et al., 2002). In addition, Di Marzio et al. (2005) performed an ecotoxicological study, where acute toxicity tests with water and sediment from Luján River were conducted with different organisms. Up to date, the most complete physicochemical monitoring study reporting the water and sediment quality of Luján River was conducted during 2004–2005 by the National Water Institute of Argentina (AABA, 2010). This report showed that the impact of anthropogenic pollution was evident in many sites of the river mainly by the presence of increased concentrations of heavy metals, organic pollutants, and coliform bacteria in surface water. In particular, in the middle reach of the river, phenolic substances range between 1.0 and 31 μg/L in Las Tropas, a place located upstream S1, whereas total hydrocarbons range between 0.3 and 1.2 mg/L in Ruta 6, a place located downstream S2 (AABA, 2010). These values are higher than (1.0 μg/L and 0.3 mg/L) the MPQ by the national law for protection of freshwater life and recreation use.

In this study, the nutrient load found in both sites of Luján River showed the presence of increased concentrations of ammonia and nitrites nearly two- and 10-fold higher than their respective MPQ values. Besides, water quality parameters of the reference sites (RSI and RSII) are in general at acceptable levels considering the criteria given in the national law for protection of freshwater life (Table I). It must be mentioned that the mean flow of Luján River is 5.37 m^3/s and that the river has a seasonal fluctuation with highest values in spring and autumn (Sánchez Caro, 2010). Therefore, the adverse impact of municipal WWTP effluents (mean flow 0.13 m^3/s) on the river water quality was modulated by the river flow during the assay period.

This study, which is the first report using an *in situ* caged fish assay conducted in the Luján River, is a biological approach that evaluates the river water quality by means of fish biomarker responses. Physiological indices, such as the CF and LSI, may be used to assess the general health and the liver metabolic activity of fish, respectively. In this study, no differences were detected in CF of *C. carpio*. This result is consistent with a previous *in situ* assay conducted with the same species in another periurban river of Buenos Aires, where no relationship was observed between CF and environmental pollution (de la Torre et al., 2000). On the other hand, *P. laticeps* fish exposed in Luján River (S1) showed an increase in CF, when compared with S2 and RSI after 14 days. High values of this index have been reported by Tejeda-Vera et al. (2007) and Smolders et al. (2004) after sampling fish from a river impacted with a sugar processing industry and after carp exposure in cages at sites impacted with industrial and WWTP effluents. These authors interpreted those results as additional food availability for test organisms. In agreement with their findings, the responses found in our study could also be interpreted in the same way. The LSI was increased in both species in cage fish exposed in Luján River; this difference was more evident in carps. Although several natural factors might affect the LSI in fish, this parameter was higher after *C. carpio* cage exposure in polluted sites of Belgium when compared with the start situation (Bervoets et al., 2009).

The toxic compounds discharged into water bodies can be incorporated into tissues of fish and then could be bioaccumulated or biotransformed, generating ROS. Biomarkers like phases I and II enzymes and oxidative stress parameters have been widely used in environmental monitoring (van der Oost et al., 2003). In this study, we used a combined battery of biomarkers, which can give useful information about the biological effect due to the exposure to complex mixture of pollutants present in Luján River.

In particular, the long-term *in situ* exposure of *C. carpio* in Luján River at both sites promoted the antioxidant defense system mediated principally by SOD, whereas CAT showed no significant changes. A simultaneous induction response in the activities of both enzymes is usually observed when exposed to pollutants (Hermes-Lima, 2004). However, other authors have reported results similar to those observed in this work, where only SOD activity was reported to be higher in polluted sites (Pandey et al., 2003; Huang et al., 2007). In agreement with our results, Camargo and Martínez (2006) did not detect changes in hepatic CAT activity when fish were exposed in cages in an urban stream in south Brazil.

The most important enzyme system responsible for oxidation processes in phase I biotransformation is cytochrome P450 monooxygenase. Among others, Hartl et al. (2007) reported the induction of this mechanism for different species in laboratory tests and monitoring. Our results indicate the induction of a CYP1A protein in exposed carps at both sites of Luján River, evidencing that phase I biotransformation processes have occurred. These results could be interpreted as a response promoted by a potential exposure to hydrocarbons previously reported in water and sediment (541 mg/kg) of areas close to the experimental sites (AABA, 2010). Similar results were reported by Hartl et al. (2007) when juvenile turbots were exposed in laboratory

assays to polluted sediments collected from Cork Harbour, Ireland.

In addition to the phase I enzyme system, phase II enzymes such as GST, which conjugate toxic compounds and their metabolites, have also been used in biomonitoring studies. An increase in GST activity has been found in various field studies with different fish species, including *C. carpio* captured at polluted sites (Pandey et al., 2003; Huang et al., 2007; Cazenave et al., 2009). We observed increased GST activity in Luján River (S1). A significant increase in liver GST activity was also detected in caged fish exposed to different sites affected by the anthropogenic influence of industrial, household, and agricultural inputs (Camargo and Martínez, 2006). In accordance with these authors, our results may indicate that these fish have detoxifying activity stimulated possibly by the presence of contaminants in the river.

Evaluation of plasma Vtg levels has been used to establish the exposure to estrogenic endocrine disruptors such as alkylphenols, alkylphenol-ethoxylates, and their most persistent metabolites (e.g., NP). Laboratory exposure of fish to NP, NPEs, or other alkylphenols has also shown a significant and time-dependent induction of Vtg concentration (Kirby et al., 2007; Genovese et al., 2011). In agreement, using Western blot, we clearly detected the expression of Vtg after 17β-estradiol or NP injections. In addition, field studies have shown increased levels of Vtg in collected (Carballo et al., 2005; Mitchelmore and Rice, 2006) and caged carp exposed (Snyder et al., 2004) to different anthropogenic inputs including discharges from WWTPs. In this study, we evidenced an induction of Vtg in carp kept in cages upstream from the municipal WWTP of Luján city (S1). In addition, the Vtg expression observed could be ascribed to the presence of NP detected in water and sediment in the Luján River during the experimental period. In this context, NP was detected in water samples obtained from S1 (0.47 μg/L) and in sediment taken from a site near WWTP (915 μg/kg dry weight) (unpublished results). In addition, phenolic substances were previously detected (AABA 2010) and in particular, the presence of *p*-nonylphenol, phenol 4-methyl and phenol 2-chloro-5-methyl was also reported by Di Marzio et al. (2005) in surface water of areas near S1 and liquid effluents of WWTP. Our results show that Western blot is a useful methodology to quickly detect the expression of Vtg in laboratory assays with specific inducers or *in situ* cage exposed fish. As shown in Figure 4, reactive bands corresponding to the apparent molecular weight of Vtg were detected using specific antibodies. However, as reported by other authors (Hennies et al., 2003; Kavanagh et al., 2004), lower molecular weight proteins were also present. The additional bands could be interpreted as breakdown products because Vtg is a highly unstable molecule. We used a protease inhibitor cocktail (Sigma P 2714) to inhibit degradation of Vtg in plasma samples from both species. However, it seems likely that this inhibitor may not have been totally effective in preventing Vtg degradation in the presence of these agents.

Long-term *in situ* exposure of *P. laticeps* in Luján River promoted mainly biotransformation phase II responses. These responses were evidenced by a marked increase in hepatic GST activity of fish kept in cages at both sites of Luján River (S1 and S2). Similar responses have been observed with other native species (*Prochilodus lineatus*) that reside in streams and polluted rivers of Argentina (Cazenave et al., 2009) and Brazil (Camargo and Martínez, 2006). In agreement with our findings, Simonato et al. (2008) did not detect any antioxidant response mediated by CAT; however, detoxification enzymes such as GST were induced after 15 days of exposure of *P. lineatus* to the water-soluble fraction of diesel oil in a laboratory assay.

BNF is a known PAH inducer of cytochrome P4501A activity in different fish species, e.g., turbot (Hartl et al., 2007) and carp (de la Torre et al., 2006). In *P. laticeps,* cytochrome P4501A induction was observed in fish injected with BNF. However, the expression of CYP1A protein in this species could not be detected in either of the experimental sites of Luján River. These results may indicate a lower sensitivity of *P. laticeps* against certain hydrocarbons that could act as CYP1A inducers that were previously reported in the river near S2 by AABA (2010). However, we cannot rule out the possibility of a lower sensitivity of recombinant CYP1A antibody against protein of *P. laticeps*. In this context, a more sensitive method evaluating mRNA induction (as reverse transcription polymerase chain reaction) could be applied, and in case of a positive result, different Western blot conditions could be assessed. Bearing in mind Vtg results, *P. laticeps* showed the presence of reactive bands of high molecular weight (130–170 kDa) only in plasma samples from fish injected with 17β-estradiol. In accordance with our results, no Vtg was detected in plasma by Mdegela et al. (2010) with an African siluriform species (*Clarias gariepinus*) captured from different sites of sewage ponds in Morogoro area.

The *in situ* approach with cages proved to be a valuable tool to measure the biological effects at different times in aquatic systems because it provides realistic exposure environments that are rarely replicated in laboratory toxicity tests. van der Oost (1998) evaluated different periods of exposure (2, 4, 6, and 8 weeks) by means of *in situ* cage assays with *C. carpio* in a heavily polluted site near Amsterdam and found that an exposure time of 4 weeks was enough to detect significant biomarker responses at the polluted site.

When short time assays (7 days) were considered, Camargo and Martínez (2006) detected differences in relative few biomarkers, including GST, after exposing *P. linneatus* in cages in an urban polluted river. In this study, we evaluated differences between two exposure

times (7 and 14 days) and found that an increase in the exposure time promoted a higher response in LSI (27%) and SOD activity (38%) in S2 fish. The same response was observed in GST activity of S1 (65%) and S2 (60%). It is important to point out that the exposure time did not promote significant differences in any of the biomarkers of caged fish in the reference site I.

Several studies have shown an increase in LPO in different fish species exposed to pollutants in field assays (Ferreira et al., 2005; Falfushynska and Stolyar, 2009). In general, this enhanced TBARS levels are attributed to enzymatic and nonenzymatic antioxidant responses that are unable to prevent oxidative damage in the liver of fish. In addition, Oakes and van der Kraak (2003) have reported high levels of hepatic TBARS in white suckers exposed immediately downstream of the effluent discharge of a municipal sewage treatment plant. In this study, unlike that found in previous reports, we observed that TBARS levels were significantly low in both species exposed in both experimental sites of Luján River. It has to be mentioned that the *in vitro* assay, conducted as positive control, showed that a known inducer of LPO (Fe^{3+}) exert response evidencing damage to lipid in the liver of *C. carpio*.

As regards the responses observed in reference sites, Figure 2 shows the RV, indicated as median and range, for each biomarker parameter. The RV bring an approximation of the values expected for each biomarker after *in situ* cage test under the assayed experimental conditions; in our case, all biomarker means of *C. carpio* fall within the expected RV range. In some parameters, the mean obtained in this study differed from the most frequent RV. Thus, the differences found in TBARS levels between the experimental and reference site could also be explained as an enhanced response in RSII. This highlights the importance of having reference data for each species when monitoring studies are conducted; therefore, RV values will be included in future field assays with *P. laticeps*. Another aspect to be considered is that, unlike standardized fish, which can be reared under controlled conditions, native organisms are usually collected from environments exposed to natural variability. This could have modulated our results, where a few biomarkers had significant responses in *P. laticeps* after *in situ* exposure. However, it should be pointed out that one of the main advantages of using native species as test organisms is their ecological relevance, as they provide more realistic information for each region.

CONCLUSION

We conclude that a multibiomarker approach with *C. carpio* and *P. laticeps* using *in situ* cage assays allowed an improved understanding of the diverse responses promoted by the exposure to contaminants present in a periurban river. This methodology could be useful in monitoring studies conducted in polluted rivers when long-term effects are assessed.

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