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## ALA-D and ALA-D reactivated as biomarkers of lead contamination in the fish *Prochilodus lineatus*

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### ARTICLE INFO

#### Article history:

Received 12 February 2010

Received in revised form

29 May 2010

Accepted 5 June 2010

#### Keywords:

ALA-D activity

Reactivation index

La Plata River

Lead

*Prochilodus lineatus*

Sewage discharges

### ABSTRACT

ALA-D activity and lead concentrations were measured in blood and liver tissues of the fish *Prochilodus lineatus*, collected from three locations along the coast of the La Plata River, Argentina. Two of them, Berazategui and Berisso, were located nearby the main ducts that discharge the urban and domestic waste disposal from Buenos Aires and La Plata cities, respectively, while the third station (Atalaya) was free of sewage discharges. For both tissues, the levels of lead in fish from Berazategui and Berisso were higher than those found in the samples from Atalaya. For blood, but not for liver, a significant negative correlation was found between ALA-activity and tissue levels of lead considering all the data. However, no good correlations were observed at each location. Therefore, an enzyme reactivation technique was optimized. The blood enzyme, but not the liver one, could be effectively reactivated with zinc (Zn<sup>II</sup>). The values of the reactivated ALA-D in samples from Berazategui and Berisso, but not from Atalaya, were significantly higher than the original values, indicating that the enzyme was actually inhibited. In addition, the reactivation index showed significant correlations with the blood lead levels. It is proposed that the reactivation index, rather than the ALA-D activity, may reflect better the extent of lead contamination, especially for field monitoring programs where many confounding factors may affect the biomarker response.

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### 1. Introduction

$\Delta$ -Aminolevulinic acid dehydratase (ALA-D) has been widely used as a reliable and sensitive biomarker of lead exposure. The enzyme specificity for this metal is considered high for most species, including mammals, aquatic organisms and bacteria (Stegeman et al., 1992; Walker et al., 2001). The enzyme may be maximally inhibited before other signs of lead toxicity become perceptible (Mayer et al., 1992). This cytosolic enzyme, also called porphobilinogen synthase (EC 4.2.1.24), is involved in the heme biosynthesis pathway and catalyzes the condensation of two molecules of  $\delta$ -aminolevulinic acid yielding porphobilinogen (PBG). The heme group is the precursor of vital macromolecules such as the hemoglobin pigment, cytochromes and chlorophyll (Jaffe, 2004), being therefore present in almost all phyla (Rocha et al., 2004).

The enzyme from animals (metazoans), fungi, archaea and some bacterium species requires zinc as a cofactor at the active site (Jaffe, 2003). However, other species may require either

magnesium or a monovalence metal ion, while others do not require any metal at the active site (Jaffe, 2004).

For the metazoan enzyme, the affinity for lead is almost 25 times greater than for zinc (Rodrigues et al., 1989; Bergdahl et al., 1998). The high specificity of lead for the ALA-D enzyme, among many other zinc proteins, may be explained by the composition of amino acids present at the active site. The yeast and mammalian enzymes contain a unique zinc-binding site with three cysteine residues (Godwin, 2001). Consequently, lead can displace zinc from the active site giving an identical molecular structure (Godwin, 2001).

In several mammalian tissues, once ALA-D has been inhibited by lead, its activity can be reactivated by adding in vitro activator agents such as dithiothreitol (DTT) and zinc (Zn<sup>II</sup>), both alone and in combination. Thus several authors have used only DTT (Granick et al., 1973; Sakai et al., 1982; Rodrigues et al., 1996), while others obtained better results with Zn<sup>II</sup> (Polo et al., 1995) or a mixture of DTT plus Zn<sup>II</sup> (Yagminas and Villeneuve, 1987) depending on the animal species and the particular tissue. In this way, it is possible to fully restore the enzyme activity. Some authors have proposed the calculation of a reactivation index that it is considered as an even better sensitive parameter to evaluate ALA-D inhibition, especially for those samples that show a high variability or when low inhibition values are observed (Rodrigues et al., 1996). The enzyme reactivation has been also investigated in both blood

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(Hodson et al., 1977; Rodrigues et al., 1989) and liver samples (Rodrigues et al., 1989; Conner and Fowler, 1994) of fish, but so far inconsistent results have been obtained.

The purposes of this work were four-fold: (i) to determine the levels of ALA-D activity in both blood and liver samples of the freshwater fish species *Prochilodus lineatus* collected from three locations along the coast of the La Plata River, Argentina; (ii) to investigate the correlations between ALA-D activity and the concentrations of lead accumulated in those tissues; (iii) to investigate if the enzyme could be reactivated in order to calculate the reactivation index and (iv) to determine if this index was a better biomarker of lead contamination than the original ALA-D activity.

The basin of the La Plata River is one of the most important in South America. The river flows into the Atlantic Ocean as a big estuary, with a highly variable width ranging from about 40 km up to 200 km at its mouth, whilst its total length is approximately 300 km (Bazán and Arraga, 1993). Along the coast there are neither mine activities nor metal smelters. Two of the sampling stations (Fig. 1), Berazategui and Berisso, are located at the vicinity of the main ducts that discharge the urban waste and sewage disposal from Buenos Aires (capital city of Argentina) and La Plata (capital city of Buenos Aires Province) without any previous treatment. Both cities and their surroundings concentrate nearly 47% of the country population. The third station, Atalaya, is located downstream and is free from sewage effluents, but it may be also subjected to minor industrial effluents and non-point agricultural runoffs. It could be argued that this last site would not be a proper election for comparative purposes. However, areas upstream to Berazategui are affected by port activities (Buenos Aires Port) and they could not be regularly sampled due to the low fish population.

Water quality data for the La Plata River are scarce. The most important monitoring program was conducted 20 years ago, sampling the principal navigation channel located about 4–5 km from the coast. According to this study, coastal areas presented

higher levels of metal contaminants (CARP-SINH-SOHMA, 1990). Lead concentrations were within the range of  $1.0\text{--}42.8\ \mu\text{g L}^{-1}$ , values equal or higher than the reference level for protecting the aquatic wildlife ( $1.0\ \mu\text{g L}^{-1}$ ) according to the recommendations for the La Plata basin (CARP-SINH-SOHMA, 1990). For this metal the highest levels were found in the samples collected near the sewage discharges at Berazategui. For other metals the ranges were  $0.1\text{--}9.1\ \mu\text{g L}^{-1}$  for cadmium (82% of the samples presented values higher than the reference level of  $0.2\ \mu\text{g Cd L}^{-1}$ );  $0.7\text{--}21.9\ \mu\text{g L}^{-1}$  for copper (a high percentage of the samples presented values higher than the reference level of  $2\ \mu\text{g Cu L}^{-1}$ ) and  $1.3\text{--}46.0\ \mu\text{g L}^{-1}$  for zinc (7% of the samples was above the reference level of  $30\ \mu\text{g Zn L}^{-1}$ ) (CARP-SINH-SOHMA, 1990).

*P. lineatus* (Class Actinopterygios, Order Characiformes) is a neotropical fish, widely distributed in the La Plata basin, where it represents 50–60% of the total ichthyomass (Oldani, 1990; Parma de Croux, 1994). This species is a bottom feeder fish that is able to incorporate contaminants present not only in the water column but also in sediment particles.

## 2. Materials and methods

### 2.1. Organism and tissues selected

Samples of *P. lineatus* were obtained from local fishermen from three sampling stations (Berazategui, Berisso and Atalaya) along coastal areas of the La Plata River, Argentina, which are presented in Fig. 1. The animals were transported alive to the laboratory in plastic aquaria (50 L) containing river water. Samplings were performed during spring 2002, autumn 2003, spring 2003 and autumn 2004 (south hemisphere) to evaluate temporal trends. However, in autumn 2003 and autumn 2004 fish were not available from the local fishermen at Atalaya and Berisso, respectively.

The Committee of Hygiene and Workplace Security, Faculty of Exact and Natural Sciences, University of Buenos Aires, has reviewed the protocols and approved the present study.

Once the animals reached the laboratory their length and weight were determined (Table 1). Only adult specimens were processed for enzyme activity and metal analysis. The sex was determined by observation of the gonads. Blood samples were extracted from the caudal vein using 5 mL heparinized syringes



Fig. 1. Map of the La Plata River showing the three sampling stations.

**Table 1**  
Mean values ( $\pm$  SD) of body length and weight of *P. lineatus*.

		Berazategui			Berisso			Atalaya		
		n <sup>a</sup>	Length (cm)	Weight (g)	n	Length (cm)	Weight (g)	n	Length (cm)	Weight (g)
Spring-2002	F	10	45.3 $\pm$ 7.4	3164 $\pm$ 1462	3	49.0 $\pm$ 1.0	2920 $\pm$ 46	3	43.8 $\pm$ 0.3	2250 $\pm$ 265
	M	8	44.1 $\pm$ 3.0	2655 $\pm$ 633	6	39.9 $\pm$ 5.2	1696 $\pm$ 433	3	51.3 $\pm$ 3.9	3050 $\pm$ 212
Autumn-2003	F	7	49.8 $\pm$ 3.1	3903 $\pm$ 852	7	40.9 $\pm$ 7.8	1921 $\pm$ 1053			
	M	3	48.5 $\pm$ 1.8	3391 $\pm$ 1265	4	39.6 $\pm$ 8.0	1513 $\pm$ 769			
Spring-2003	F	8	47.5 $\pm$ 4.3	3254 $\pm$ 1075	8	40.6 $\pm$ 3.8	1925 $\pm$ 619	4	41.7 $\pm$ 4.7	1720 $\pm$ 627
	M	6	44.7 $\pm$ 3.7	2866 $\pm$ 616	3	37.3 $\pm$ 3.1	1467 $\pm$ 633	3	41.0 $\pm$ 7.1	1300 $\pm$ 283
Autumn-2004	F	4	49.4 $\pm$ 1.6	3490 $\pm$ 308				3	41.6 $\pm$ 2.4	1732 $\pm$ 243
	M	4	43.6 $\pm$ 4.1	2628 $\pm$ 957				5	42.7 $\pm$ 3.0	1940 $\pm$ 494

F: Female; M: Male.

<sup>a</sup> Number of samples.

(0.30 mg heparin per mL of blood). One aliquot was kept in liquid nitrogen until the enzyme activity was measured. The other aliquot was reserved for hematocrit determinations and another was used for lead analysis. Then, the animals were killed by excision of the spinal cord behind the operculum. Liver tissues were dissected at 0–2 °C.

## 2.2. ALA-D activity

Blood samples (0.1 mL) were treated with distilled water (0.65 mL) and incubated at 38 °C during 15 min to break red cell membranes (Berlin and Schaller, 1974). Then, 0.5 mL of buffer substrate, containing 10 mM  $\delta$ -aminolevulinic acid dissolved in 50 mM phosphate buffer at pH=6.2 (prepared just before using) were added.

The whole liver was washed in 50 mM Tris-HCl buffer at pH=7.4, dried on filter paper and weighed. About 5 g were used for lead analysis. Another portion was homogenized in 50 mM Tris-HCl buffer at pH=7.4 (ratio 1:3 w/v). Homogenates were centrifuged at 11,700g during 20 min at 2 °C and the pellets discarded. Supernatants were kept in liquid nitrogen for the analysis of the enzyme activity. The liver ALA-D activity was measured in an aliquot of 0.2 mL of the supernatants and adding 0.5 mL of buffer substrate.

For both blood and liver samples, the tubes were incubated for 1 h at 38 °C. Finally, 0.5 mL of trichloroacetic acid were added to stop the reaction (final concentration=10%). The tubes were centrifuged at 4000 rpm for 10 min. An aliquot of 1 mL of the supernatant was transferred to another tube where 1.0 mL of Ehrlich's reagent was added. The Ehrlich's reagent was prepared by dissolving 0.24 g of *p*-dimethylaminobenzaldehyde in 1.92 mL of perchloric acid and 12 mL of glacial acetic acid. The reaction product (PBG) was measured at  $\lambda=555$  nm in a Metrolab UV/visible spectrophotometer. Each sample was analyzed at least by duplicate.

An enzymatic unit (U) of ALA-D was defined as the amount of enzyme catalyzing the formation of 1 nmol of product (PBG) in 1 h at 38 °C. The blood enzymatic activity (EA) was calculated as:

$$EA = \frac{U}{\text{Hematocrit}} \times 100$$

The hematocrit was determined by the capillary method. The EA for liver samples was expressed as U per mg of protein. Protein concentrations were determined following the method of Lowry et al. (1951) using bovine serum albumin as standard.

## 2.3. ALA-D enzyme reactivation

The ability of DTT, Zn<sup>II</sup> and binary mixtures of DDT plus Zn<sup>II</sup> as enzyme reactivator agents was tested in both blood and liver samples. Both tissues were obtained from 6 adult fish collected in Berazategui (34.4  $\pm$  2.0 cm, 1040  $\pm$  151 g) and were isolated as described in Section 2.1. Different aliquots were added to the samples from stock solutions containing either 20 mM DTT (Sigma-Aldrich, Argentina) or 500  $\mu$ M Zn<sup>II</sup>, prepared using Zn (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub> (Merck, Argentina). Then, the tubes were incubated for 15 min at 38 °C prior to the addition of the buffer substrate. The enzyme activity was determined as previously described. Each sample was analyzed at least by duplicate. Once the technique was optimized, ALA-D was reactivated from blood samples by adding only Zn<sup>II</sup> (final concentration 15  $\mu$ M).

The reactivation index (RI) was calculated using both values of enzyme activity, the original ALA-D activity and the reactivated ALA-D(r) activity, as

$$RI = \frac{ALA-D(r) - ALA-D}{ALA-D(r)} \times 100$$

## 2.4. Lead analyses

Blood samples were treated with 0.05% Triton X-100 and 0.5% phosphate solution. The metal was quantified by electrothermal atomic absorption spectrophotometry (AAS). Values of metal concentrations were expressed as  $\mu$ g Pb dL<sup>-1</sup>. The detection limit was 0.05  $\mu$ g Pb dL<sup>-1</sup>. Each sample was analyzed at least by duplicate. Standard solutions for AAS were prepared from a stock solution containing 1000 mg Pb L<sup>-1</sup> (CertiPUR®, Merck Company, Argentina).

Approximately 1–2 g (wet weight) of liver tissue was placed in acid prewashed borosilicate tubes. The digestion procedure was performed immediately after dissection in the presence of 5–10 mL of ultrapure concentrate nitric acid (Merck Laboratories, Argentina) at 100–120 °C until the complete destruction of the organic material. Then the samples were diluted to a final volume of 5 mL with 1% (v/v) ultrapure nitric acid, and centrifuged to remove any residue. Each tissue was analyzed at least by duplicate. For each 10 samples, a blank was performed and processed simultaneously. Metal concentrations were measured in an atomic absorption spectrophotometer with background correction, applying the method of direct flame atomization in an air-acetylene flame. Values of metal accumulation were expressed as  $\mu$ g metal per g wet tissue. The detection limit was 0.3  $\mu$ g Pb g<sup>-1</sup>.

All the glassware were prewashed with 5% nitric acid, thoroughly rinsed with double-distilled water, and dried. Blank values were negligible for lead. Five certified reference materials (TM-25, TM-15, TMDA-61, TMDA-62 and TMDA-64, from the National Water Research Institute of Canada) were measured. In all cases QA/QC recoveries were within the range of 97–103%. To check the accuracy of the analyses, the standard addition method was used by adding a known lead concentration to the liver samples before digestion.

## 2.5. Statistical analyses

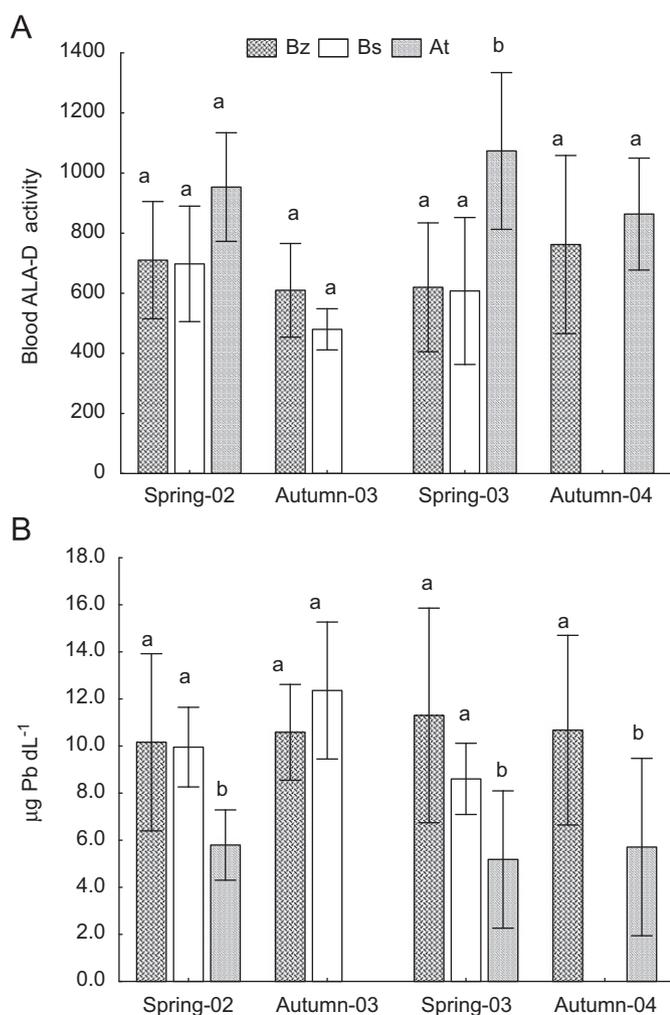
Except for the correlation coefficient, all the statistical analyses were performed using Statistica Program, version 7.0 (StatSoft Company, USA). Data of ALA-D activity and lead content followed a normal distribution (Anderson-Darling's test) but had heterogeneous variances (Bartlett's test). Therefore, comparisons between both sexes and among sampling stations were performed using the non-parametric Kruskal-Wallis test (Sokal and Rohlf, 1997). Pearson's correlation coefficients were calculated using the Excel® software package (Microsoft, USA). The effect of the different activator agents on ALA-D activity was analyzed by ANOVA test. Comparisons between ALA-D activity and reactivated ALA-D activity were also tested by ANOVA. In all cases the significance level was set at  $p=0.05$ .

## 3. Results

### 3.1. ALA-D activity and lead levels in blood samples of *P. lineatus*

Values of ALA-D activity in blood samples of *P. lineatus* collected from the three sampling stations are shown in Fig. 2A. The data for both sexes were pooled, since no significant differences were observed between male and female fish ( $p > 0.05$ ) in any of the sampling periods.

In spring 2002, autumn 2003 and autumn 2004 the values of ALA-D activity did not differ among the sampling stations



**Fig. 2.** (A) ALA-D activity (mean values  $\pm$  SD) in blood samples of *P. lineatus* collected in Bz: Berazategui, Bs: Berisso and At: Atalaya. (B) Values of lead concentrations ( $\mu\text{g Pb dL}^{-1}$ ) in blood samples. Different letters indicate significant differences among the sampling stations but only within each sampling period (Kruskal–Wallis,  $p < 0.05$ ).

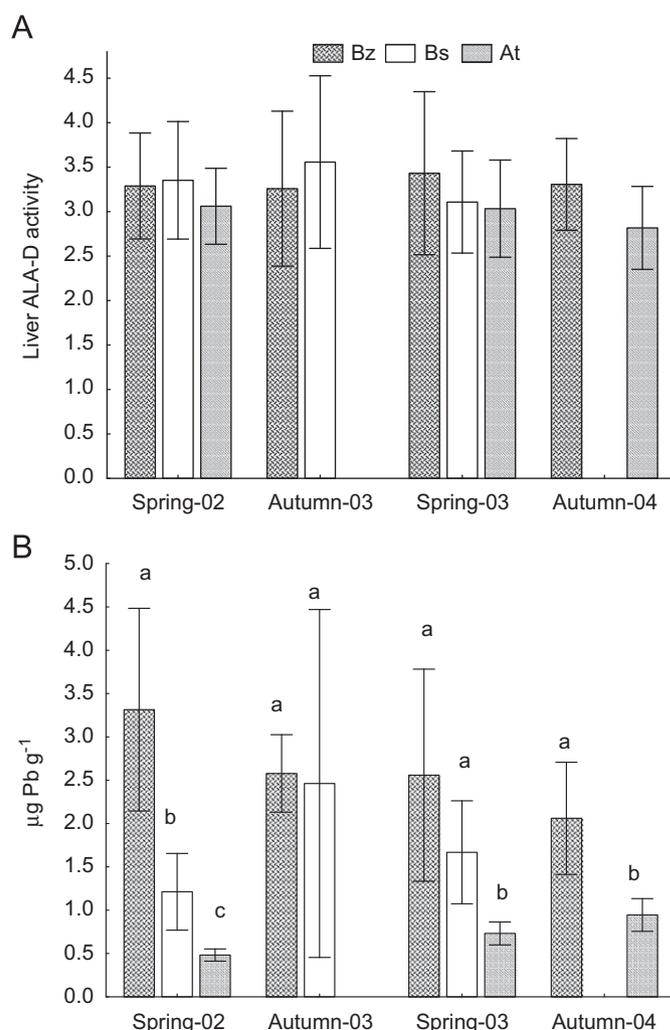
( $p > 0.05$ ). Only in spring 2003 higher values of enzyme activity were observed in the samples collected from Atalaya in comparison with those obtained from the areas of influence of the sewage discharges (Berazategui and Berisso) ( $p < 0.05$ ).

The values of lead concentrations in blood samples are presented in Fig. 2B. Since no significant differences were observed between male and female fish ( $p > 0.05$ ), the data were pooled. During the periods studied, samples from Berazategui and Berisso did not show significant differences between them ( $p > 0.05$ ) but they presented higher levels of metal concentrations than those from Atalaya ( $p < 0.05$ ).

### 3.2. ALA-D activity and lead levels in liver samples of *P. lineatus*

The results of enzyme activity and lead concentrations in liver samples of *P. lineatus* are presented in Fig. 3A and B, respectively. Similarly to what was found for the blood samples, no significant differences by sex were found either in ALA-D activity or lead concentrations ( $p > 0.05$ ); therefore, in both cases the data were pooled.

The values of enzyme activity (Fig. 3A) did not present significant differences over the period studied for any of the



**Fig. 3.** (A) ALA-D activity (mean values  $\pm$  SD) in liver samples of *P. lineatus* collected in Bz: Berazategui, Bs: Berisso and At: Atalaya. (B) Values of lead concentrations ( $\mu\text{g Pb g}^{-1}$  wet tissue) in liver samples. Different letters indicate significant differences among the sampling stations but only within each sampling period (Kruskal–Wallis,  $p < 0.05$ ).

sampling stations ( $p > 0.05$ ). Instead, higher levels of lead were observed in liver samples of fish collected in Berazategui and Berisso than those observed in the samples from Atalaya ( $p < 0.05$ ) (Fig. 3B).

### 3.3. Correlations between ALA-D activity and lead concentrations

Pearson's correlation coefficients between the enzyme activity and the levels of lead for each tissue in each sampling station are presented in Table 2.

In general, for each sampling station the correlation coefficients were very low, indicating the lack of any significant correlation, except for the blood samples of fish from Berazategui. However, when all the data were pooled a significant negative correlation was found for blood samples ( $p < 0.05$ ) but not for the liver ones ( $p > 0.05$ ).

### 3.4. Reactivation of ALA-D activity

The effects of variable concentrations of DTT, Zn<sup>II</sup> and Zn<sup>II</sup> plus DTT on ALA-D activity are presented in Fig. 4 for blood and liver samples.

Within the ranges of 0–2.4 mM for blood samples and 0–0.024 mM for liver samples, DTT did not modify ALA-D activity ( $p > 0.05$ ). Instead, higher concentrations induced an inhibitory effect ( $p < 0.05$ ). In the presence of  $Zn^{II}$ , the range of highest enzyme activity corresponded to 10–20  $\mu M$  ( $p < 0.05$ ). However, the liver enzyme could not be reactivated by any  $Zn^{II}$  concentration.

The effect of binary mixtures of  $Zn^{II}$  plus DTT was tested selecting a constant value of DTT that did not induce enzyme inhibition (0.24 and 0.024 mM DTT for blood and liver samples,

respectively) and varying  $Zn^{II}$  concentrations. Within the range of 0–40  $\mu M$  of  $Zn^{II}$  the values of the blood enzyme activity remained unchanged ( $p > 0.05$ ), but at higher levels of  $Zn^{II}$ , the activity significantly decreased ( $p < 0.05$ ). A similar pattern was observed for the liver enzyme.

Once it was shown that the blood enzyme activity could be reactivated by  $Zn^{II}$  only (15  $\mu M$ ), the technique was applied to determine all the reactivated ALA-D values for blood samples. The results are shown in Table 3. The reactivation index is also included.

Clearly, the values for the reactivated enzyme in the blood samples collected in Berazategui and Berisso were higher than the values for the non-activated enzyme ( $p < 0.05$ ). However, the values for the samples collected in Atalaya were not significantly different compared to the ALA-D activities initially recorded ( $p > 0.05$ ).

### 3.5. Reactivation index versus lead concentrations in blood samples

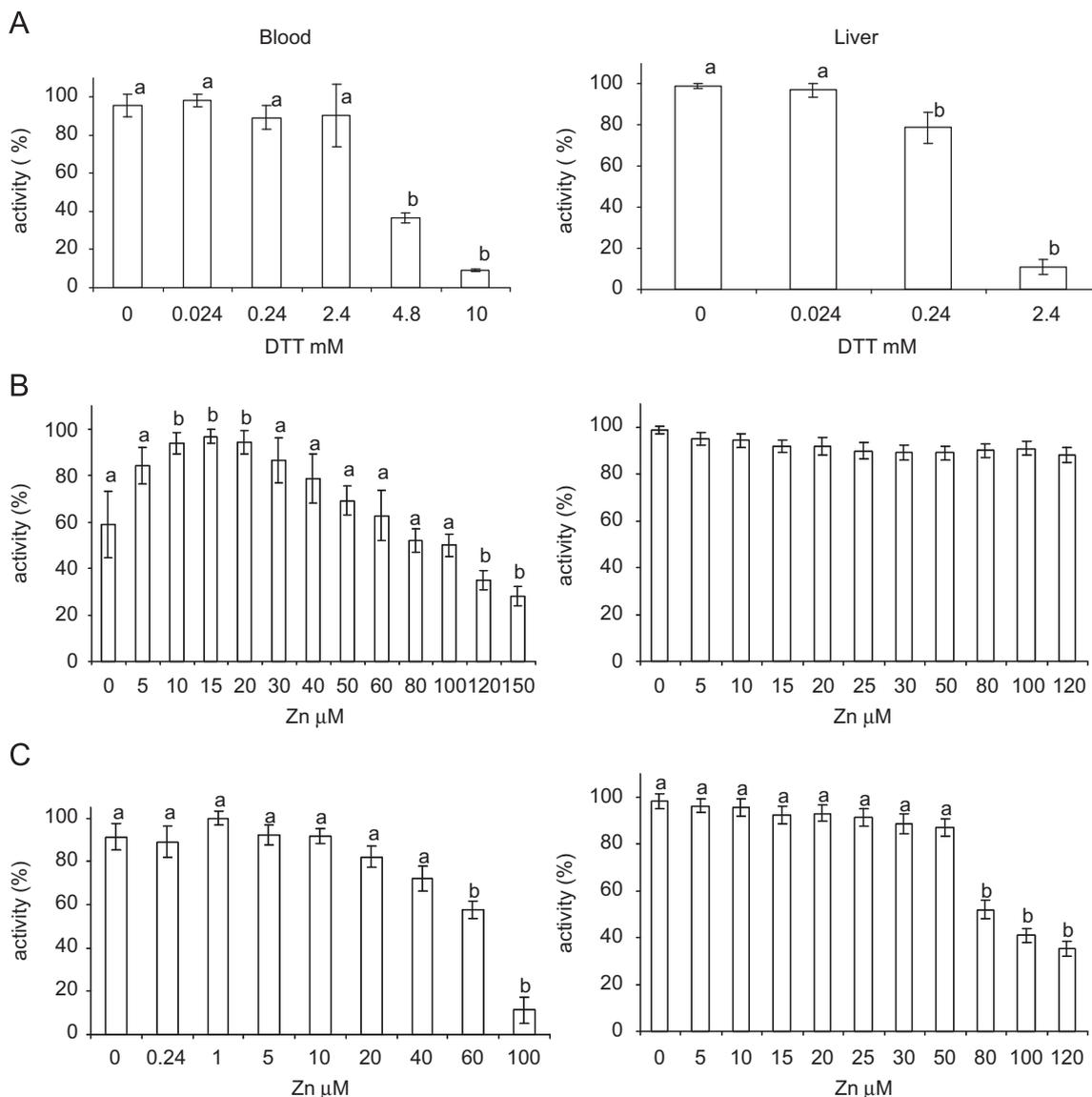
The reactivation index in blood samples versus the blood lead concentrations is presented in Fig. 5A and B for Berazategui and Berisso, respectively.

**Table 2**

Pearson's correlation coefficients between ALA-D activity and lead concentration in tissues of *P. lineatus*.

	Blood tissue	Liver tissue
Berazategui	-0.34*	-0.006
Berisso	-0.28	0.15
Atalaya	-0.16	0.19
All sampling stations	-0.51*	0.16

\* Indicates significant differences at  $p < 0.05$ .



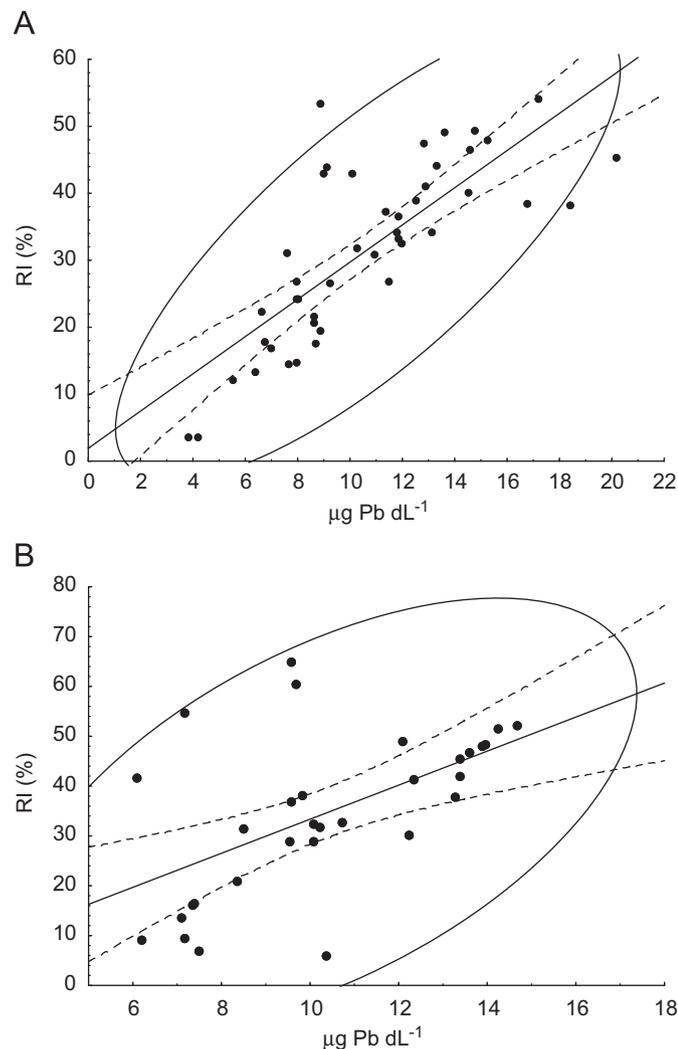
**Fig. 4.** Effect of activator agents on ALA-D activity in blood and liver samples of *P. lineatus*: (A) different DTT concentrations, (B) different  $Zn^{II}$  concentrations, (C) binary mixtures containing 0.24 or 0.024 mM DTT for blood and liver samples, respectively, plus different  $Zn^{II}$  concentrations. Different letters indicate significant differences with respect to the original ALA-D activity (no activator added) (ANOVA,  $p < 0.05$ ).

**Table 3**  
Values of reactivated ALA-D activity (ALA-Dr) (mean values  $\pm$  SD) and reactivation index (RI) for blood samples of *P. lineatus*.

	Berazategui		Berisso		Atalaya	
	ALA-D(r)	RI	ALA-Dr	RI	ALA-D(r)	RI
Spring 2002	1014 $\pm$ 206*	30 $\pm$ 16	974 $\pm$ 219*	29 $\pm$ 7	1065 $\pm$ 235	10 $\pm$ 7
Autumn 2003	895 $\pm$ 148*	32 $\pm$ 11	909 $\pm$ 146*	47 $\pm$ 5	ND	
Spring 2003	955 $\pm$ 229*	35 $\pm$ 13	840 $\pm$ 216*	27 $\pm$ 21	1260 $\pm$ 279	11 $\pm$ 7
Autumn 2004	1105 $\pm$ 429*	29 $\pm$ 11	ND		956 $\pm$ 203	10 $\pm$ 6

ND: not determined.

\* Indicates significant differences with original ALA-D values (ANOVA,  $p < 0.05$ ).



**Fig. 5.** Regression lines between the reactivation index (RI) and the levels of lead concentrations in blood samples of *P. lineatus* collected in (A) Berazategui ( $r=0.78$ ;  $p=0.0000$ ) and (B) Berisso ( $r=0.59$ ;  $p=0.0012$ ).

In both sampling stations, very good correlations were found, which corresponded to significant linear regressions ( $p < 0.001$ ).

#### 4. Discussion

Levels of lead in both blood and liver samples of fish collected in Atalaya were always lower than the values found in fish from

Berazategui and Berisso, without presenting significant temporal variations. The results suggest that the fish had actually been exposed to and had incorporated the metal. This trend is in agreement with the highest concentration of lead previously recorded in water samples collected near the sewage discharges at Berazategui (CARP-SINH-SOHMA, 1990).

Based on the metal bioaccumulation data it could be expected that the ALA-D activity in samples from Berazategui and Berisso had lower values than samples from Atalaya. However, in general these values did not present significant differences among the sampling stations or the sampling periods. When comparing blood samples of fish from Berazategui and Berisso with those from Atalaya, significant decreases in the enzyme activity were only observed in spring 2003. A significant negative correlation coefficient was observed between all the data of blood ALA-D activity and blood lead content, but very low values were observed when they were calculated at each sampling station. However, in all the liver samples, the ALA-D activity remained unchanged over the period studied. In this way, it is not surprising to find that the data of enzyme activity did not positively correlate with the lead content in any case.

Biomarkers should meet several criteria before using them in field monitoring programs (Mayer et al., 1992; Kammenga et al., 2000; van der Oost et al., 2003). One of the most important requisites when selecting a biomarker is that the response should be related to the environmental or internal contaminant levels. ALA-D is a specific biomarker for lead exposure and previous studies have shown very good negative correlations between ALA-D activity and blood lead concentrations in humans exposed to the metal (Peakall, 1994; Gurer-Orhan et al., 2004). Similarly, good correlations have been reported in blood samples from several fish species (Hodson et al., 1977, 1978; Nakagawa et al., 1995a, 1995b, 1997), and in tissues of freshwater invertebrates (Verrengia Guerrero et al., 1997; Aisemberg et al., 2005).

However, most of the animal data have been obtained by laboratory bioassays exposing the organisms to the metal (usually the only contaminant) under normalized conditions. In most cases the bioindicator organisms have been also reared under normalized laboratory conditions. Such controlled conditions are by far much less complex than natural environments. Instead, in field situations, where the animals are exposed chronically, significant correlations are not always so evident, at least for all the tissues analyzed (Schmitt et al., 1984, 1993, 2007; Vanparys et al., 2008).

Most of the field monitoring programs based on biomarker determinations considers upstream–downstream comparisons to assess the effect of a specific point source. Alternatively, the samples can be collected along a well characterized pollution gradient, obtaining significant correlations between ALA-D inhibition and lead levels (Vanparys et al., 2008). However, these approaches are not easily applicable to the La Plata River due to the particular characteristics of this system. The river is practically as wide as large. In addition, it is subjected to the influence of the marine currents that are opposite to the river currents, so the concept of upstream–downstream is not meaningful.

Although lead concentrations in samples of *P. lineatus* collected from Berazategui and Berisso were higher than those from Atalaya, the values of ALA-D activity did not clearly reflect the bioaccumulation pattern. It could be suggested that as a consequence of the high variability in the values of ALA-D activity it could mask any significant differences among the sampling sites.

It is not unusual to find a greater variability in biomarker responses when measuring in biological tissues collected from natural environments in comparison with those measured in samples of organisms that were exposed under laboratory conditions. In field situations inter-individual variations

may be due to multiple factors, such as an increased genetic heterogeneity, diversity of age, size, nutritional status, sex and also to the influence of other natural and anthropogenic variables (Mayer et al., 1992).

The environmental factors (such as water temperature, salinity, ionic strength and food availability) should elicit the same influence in the fish collected from a particular area and at a given period. Therefore, the biological and physiological variables could play the major role influencing the variability. The condition factor is a morphometric index that may be used to estimate the physiological status of the animals (Goede and Burton, 1990). However, the condition factors for *P. lineatus* did not present significant differences among the three sampling stations over the period studied and the values were not influenced by the sex of the animals (Lombardi et al., 2010).

For both blood and liver samples, the values of ALA-D activity and the lead concentrations did not present significant differences by sex. Few studies have investigated the influence of sex on ALA-D activity. According to the available reports, no sex associated differences were observed in blood levels of this enzyme from several bird species (Dieter et al., 1976; Finley et al., 1976; Grue et al., 1984).

Accordingly, the inter-individual variations together with other environmental factors, may account for the high enzyme variability rather than the physiological status of the animals or their sex. Since the data of ALA-D activity in *P. lineatus* was difficult to interpret, the enzyme reactivation technique was tested.

The assays showed that Zn<sup>II</sup> alone (in the range of 10–20 µM) was the best activator agent. Instead, in the presence of either DTT or mixtures of DTT plus Zn<sup>II</sup> the ALA-D activity remaining unchanged or even decreased depending on the concentrations. According to our best knowledge, this is the first report where the ALA-D activity could be successfully reactivated in fish blood. The optimal concentrations of Zn<sup>II</sup> needed for reactivating the blood enzyme in *P. lineatus* were considerably lower than the values reported for mammals, where the range varies between 90 and 100 µM Zn<sup>II</sup> for blood samples (Granick et al., 1973; Finelli et al., 1974; Yagminas and Villeneuve, 1987). On the contrary, at this range an inhibitory effect was observed in the blood samples of *P. lineatus*.

The results also showed that the blood enzyme, but not the liver one, could be effectively reactivated. The different behavior observed for blood and liver samples may be due to the fact that each tissue presents a characteristic isoenzyme. In fact, both enzymes have different affinity constants and optimal pH ranges (Lombardi, 2008) that may explain the different sensitivity to lead. Thus, the usefulness of the reactivation technique would be limited by the characteristics of the isoenzyme present in a particular tissue of a given organism. This may explain the inconsistent results for reactivating the ALA-D enzyme found in other fish species.

Interestingly, the values for the reactivated enzyme activity did not present any significant differences among the sampling stations or the sampling periods. In addition, blood samples of *P. lineatus* collected from another aquatic system (located about 1000 km to the north from Buenos Aires city) with lower levels of metal accumulation (range <0.1–4.4 µg Pb g<sup>-1</sup> wet weight) presented similar values of ALA-D activity (1229 ± 31 EA) (Lombardi, 2008). Therefore, the reactivated enzyme values may be considered as the maximal basal levels of ALA-D activity for *P. lineatus*.

On the other hand, the reactivation index, that represents the percentage of enzyme inhibition, indicated that the blood enzyme of fish collected in Berazategui and Berisso was actually inhibited (from 27% up to 49%), but no inhibition was observed in the

samples from Atalaya. In addition, significant correlations were found between the reactivation index and the blood lead content.

## 5. Conclusions

From the original data of ALA-D activities alone it could be wrongly concluded that for most of the samples the levels of lead bioaccumulated by *P. lineatus* were not high enough as to inhibit the activity of the enzyme present in blood and liver tissues. In fact, this may be true for the liver isoenzyme but not for the blood one. In this last tissue, Zn<sup>II</sup> was able to restore the enzyme activity. By using the reactivated ALA-D values and the reactivation index it could be shown that the enzyme was actually inhibited in the samples from Berazategui and Berisso, but not in those collected from Atalaya.

Therefore, it is proposed that the reactivation index may constitute a more reliable and sensitive biomarker of lead exposure, especially for complex field conditions where the animals are chronically exposed to the metal in the simultaneous presence of many other natural and anthropogenic chemicals and no reference unpolluted sites could be identified.

The higher lead concentrations together with the inhibition of ALA-D provide supporting evidence that the untreated sewage discharges are sources of lead contamination. These point source emissions may have a negative impact on the fish population.

## Acknowledgments

This work was partially supported by Grants X-147 and X-233 from the University of Buenos Aires and PICTR 2002-00203 from the Agencia Nacional de Promoción Científica y Tecnológica. Our gratitude to the local fishermen.

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