THE EFFECT OF SUBLETHAL CONCENTRATION OF LEAD ON DELTA-AMINOLEVULINIC ACID DEHYDRATASE ACTIVITY AND HEMATOLOGICAL PARAMETERS IN THE JAVANESE CARP *Puntius gonionotus* (BLEEKER)

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ABSTRACT

The activity of the erythrocyte delta-Aminolevulinic acid dehydratase (ALA-D) of carp, *Puntius gonionotus*, was measured under a variety of lead (Pb) exposure conditions. A four-week exposure of carp to Pb concentrations of 10 ppb - 300 ppb strongly inhibited their ALA-D activities with increasing Pb concentrations. When carp were exposed to Pb concentration of 300 ppb, depressed activity of ALA-D became visible after only 2 days, and this activity declined further with increasing exposure of seven weeks in Pb-free water. These characteristics indicate that this enzyme can be useful as a short-term indicator of Pb pollution. In the hematological work it was found that when the fish were exposed for 4 weeks to the highest Pb concentration (300 ppb), they suffered from anaemia, with decreasing Hematocrit values. There was no significant ($P>0.05$) interaction between Pb concentration and either lactate level or blood glucose.

KEYWORDS: Lactate, glucose, Delta Aminolevulinic Acid (ALA-D), lead, sublethal.

INTRODUCTION

The contamination of natural waters by lead (Pb) is mostly caused by a variety of anthropogenic activities related to an increase in mining operations and industrial uses of this metal. Of the five most common and toxic heavy metals in the atmosphere (lead, mercury, cadmium, arsenic and selenium), Pb contributes 87.4% of the total atmospheric emissions from both anthropogenic and natural sources. The metal itself is used in many ways in sheet, foil or pipe forms. Alloys with antimony and/or tin are used for bearings, printing type, accumulator plates, solder, bullets and shot. White lead (PbO) and lead carbonate ($PbCO_3$) are used in pottery glazing, and red lead (Pb_3O_4) is a protective primer for steel. Most of the Pb in the atmosphere is from the consumption of leaded gasoline (Nriagu & Pacyna, 1988), in which the organic compound, lead tetraethyl, is an "anti-knock" agent added to high octane petroleum. It is converted into a fine particulate inorganic form of Pb during combustion and discharged into the atmosphere.

Adverse effects caused by Pb poisoning in fishes have been reported with reference to both hematological and biochemical variables (Jackim, 1979; Hodson, 1976). Among the clinical symptoms described in fishes are anaemia, basophilic stippling of erythrocytes, muscular atrophy, the degeneration of the caudal fin and lordoscoliosis (Dawson, 1935).

Water quality criteria for heavy metals are often based on the response of fish to long-term metal exposures. However, studies of these responses may take several years and provide information on only one metal and one species of fish in one situation. A short-term indicator

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of sublethal Pb toxicity would provide a faster, more economical way of extending these results to other fish or other situations.

In blood, Pb is mainly (90%) bound to erythrocytes (Lauwerys & Hoet, 1993), so whole blood should be taken for analysis. Biological tests have been proposed for the detection of excessive exposure to Pb, mainly based on the interference of Pb with several enzymatically controlled stages of the heme synthesis pathway. In vitro, Pb can inhibit the enzyme, (-Aminolevulinic acid synthetase, but in vivo it seems that an induction of the activity of this enzyme is more likely to be produced (Meredith *et al.*, 1978). This action could result from a depression of the enzyme synthesis due to the decreased amount of heme. In fish, the sensitivity of delta ALA-D to lead poisoning had been demonstrated through three features: as a reduction of the ALA-D activity in liver and kidney of winter flounder, *Pseudopleuronectes americanus* and *mummichog*, *Fundulus heteroclitus* (Jackim, 1979); as depressed activity of red blood cell ALA-D in rainbow trout, *Salmo gairdneri*; brook trout, *Salvelinus fontinalis*; goldfish, *Carassius auratus*; and *pumpkinseed*, *Lepomis gibbosus* (Hodson, 1976); and in the erythrocytes, spleen, and renal tissue of rainbow trout, *Salmo gairdneri* (Johansson-SjÜbeck & Larsson, 1979). Furthermore, the absence of erythrocyte ALA-D inhibition in fish exposed to cadmium, copper, zinc and mercury, indicated that this enzyme is quite specifically inhibited by Pb (Rodrigues *et al.*, 1989).

In this work, the activity of (-Aminolevulinic acid dehydratase in the blood of Pb exposed and unexposed fish will be measured to determine whether this is a useful technique for quick identification of harmful Pb exposures.

MATERIALS AND METHODS

Test Fish

The fish, Javanese carp (*Puntius gonionotus* Bleeker), were obtained from the hatchery unit of Universiti Pertanian Malaysia, Serdang Selangor. They measured 20.0±0.90cm in total length and 28.0±0.57g in weight. They were acclimatized for 10 days before starting the experiment. The fish were fed once a day with a commercial diet, and the holding tanks were cleaned daily shortly after feeding.

Test Water

Lead nitrate (reagent grade) was dissolved in 0.1N nitric acid solution, and a stock solution of 10mg/mL (as Pb) was made. The stock solution was diluted with seasoned water, and different concentrations of Pb for three types of exposure tests were prepared.

Basic Method for Exposure Tests

The test tanks were quadrangular aquaria (150x30x30cm^3), made of glass and subdivided into small interconnected cells (30x30x21cm^3) by a plastic net, to let water pass freely through all cells. The basic exposure test was conducted by circulation of test waters through test tanks by aeration and replacement of test waters at 48 hour intervals. The test was conducted at room temperature (26-29°C). The fish were fed to satiation with commercial diet 5 hours prior to the replacement of the test waters. The pH of the test waters was within the range of 7.0-8.0 throughout the test period. Dissolved Pb from the test tanks was analysed for the actual concentration using an atomic absorption spectrophotometer (AAS-Model Shimadzu-670).

Exposure Test - 1

In order to examine the relationships between ALA-D activity and water Pb concentration, water Pb concentration and blood Pb concentration, fish were exposed for 4 weeks to Pb concentrations of 10, 75 and 300ppb. A control was provided which had no Pb content.

The experiment was carried out in the four different test tanks (as mentioned earlier) each containing 50 litres of test solutions where 1 fish was placed in each subdivision of the tank. The experiment was done in duplicate and repeated twice.

Exposure Test - 2

Fish were exposed for 4 weeks to water of Pb concentration of 300ppb and a control (no Pb). The test began with two test tanks per each of control water and Pb contaminated water - exposed groups. One fish from each of the test tanks of each group was randomly collected for blood sampling. The remaining fish were redistributed randomly to each subdivision of the test tanks. Subsequently, the above procedure was repeated for every blood sampling.

Exposure Test - 3

To examine recoveries of ALA-D activities and Pb concentrations in the blood of fish, experimental fish were transferred to Pb-free water and kept for 7 weeks after the fish had been exposed for 4 weeks to water Pb concentration of 300ppb. The exposure test began with two test tanks each containing 5 subdivision per each group of control - water and Pb contaminated water exposed groups. After 7 weeks, fish from each of the test tanks of each group was randomly collected for blood sampling.

Collection of Blood

Each fish was anesthetized by immersion in tricaine methanesulfonate (MS-222) for several minutes before blood and tissue samples were collected. Blood was drawn by caudal peduncle puncture using a heparinized 3-ml syringe equipped with a 20 - gauge needle and each fish was then weighed and measured. The blood was dispensed from the syringe into a heparinized test tube and measurement of Hematocrit (Ht), ALA-D activity and Pb concentrations were made.

Measurement of ALA-D Activity

Blood was assayed for ALA-D activity by a method modified from Granick *et al.* (1973). Two samples of 75µL of blood were drawn by caudal peduncal puncture using a chilled, heparinized capillary tube. One tube was capped and centrifuged to measure the micro-Hematocrit. Blood from the second tube was immediately transferred to parafilm and 20µL aliquots were added to each of three disposable centrifuge tubes at room temperature. The first was a blank which contained 100µL of 0.2% Triton X-100 in 0.1M, pH 6.2 phosphate buffer. The second and the third tubes were duplicated tests and contained 100 µL of 0.2% Triton X-100 and 670µg/mL of Aminolevulinic acid in 0.1M, pH 6.2 phosphate buffer. The tubes were capped and the sample mixed and incubated at 37°C for 1 hour. The reaction was stopped with 300µL of an aqueous solution of 4.0g of trichloroacetic acid and 2.7g of mercuric chloride per 100mL. Each tube was centrifuged at 2500g for 5 minutes and 350µL of supernatant was transferred to a glass test tube. The amount of porphobilinogen produced in the reaction was measured by adding 300µL of modified Ehrlich's Reagent to each test tube. This reagent consisted of 168mL glacial acetic acid; 40 mL of 70% perchloric acid, 4.0g of p-dimethyl amino benzaldehyde, 0.7g mercuric chloride, and 12 mL distilled water. The reagent was prepared fresh every time. After 15 minutes of color development, absorbance of each test solution was read against the appropriate blank at 553nm using a spectrophotometer. The ALA-D activity was expressed as units per milliliter of red blood cells per hour using the formula:

$$Activity = \frac{D \times A}{T \times H}$$

where: D = dilution coefficient = 50
A = absorbance after 1 hour
T = incubation time (1 hour)
H = hematocrit ÷ 100

Lead Analysis

200µL of nitric acid (61%) and 200µL of hydrogen peroxide were added to 200µL of collected blood. The mixture was wet - digested on the hot plate at about 140°C until dried. The dried digestate was dissolved with 4mL of 0.1N nitric acid solution. Water Pb concentrations were determined by adding 0.8mL of concentrated nitric acid to 100mL of each test water and the test waters were stored at room temperature until analysis. The relationships between blood and water Pb were determined using statistical analysis and Pb concentrations were determined using Atomic Absorption Spectrometry (Model Shimadzu-670).

Hematological Parameters Analysis

Blood samples were taken within 35 seconds after collecting the fish from the tank. Hematocrit (Ht) was measured using capillary tubes filled with blood and centrifuged at 11000r/min for 6 minutes. Hemoglobin, glucose and lactase levels were measured based on calorimetric determinations using Sigma Diagnostics Commercial Kits.

i) Total Hemoglobin Analysis

The total hemoglobin analysis was determined by adding 5.0mL Drabkin's Solution to the blank and test samples. To each of the samples, 20µL whole blood was added and allowed to stand for 15 minutes at room temperature (26°C). The absorbance was read at a wavelength 540nm. Total hemoglobin concentration (g/100mL), was determined using the calibration curve.

ii) Glucose Analysis

For the glucose analysis, all test tubes were labelled as either BLANK, STANDARD or TEST. The blank test tube had 0.1mL distilled water added and the STANDARD had 0.1mL Glucose Standard Solution Catalogue No. 635-100 added. The TEST tubes had 0.1mL plasma, then 5.0mL O-Toulidine Reagent, Catalogue No. 635-6 added. It was mixed by lateral shaking. After shaking, all tubes were placed in a vigorously boiling water bath for exactly 10 minutes. All tubes were quickly removed and cooled to room temperature by placing in tap water for approximately 3 minutes. The contents of the tubes were then transferred to cuvets, and the absorbance of STANDARD and TEST at 635±15nm were recorded using the blank as reference. All reading were completed within 30 minutes. Glucose concentrations were calculated using the formula;

$$Glucose\ (mg/mL) = \frac{A\ Test}{A\ Standard} \times 100$$

iii) Lactate Analysis

Lactate Reagent Solution was used for this analysis. To each sample tube, 1.0mL of Lactate Reagent Solution was added. To tubes labelled as STANDARD, 10µL of Lactate Reagent Solution, Catalogue No. 826-10 was added. 10µL of blood plasma was added to tubes labelled TEST. All tubes were incubated for 5 to 10 minutes. The absorbance (A) of STANDARD and TEST vs. BLANK as reference were recorded at 540nm. The reading was completed within 10 minutes following incubation. Lactate concentration was calculated using the following formula:

$$Lactate\ (mg/100\ mL) = (40.5)\ (Au)\ (C)$$

where: Au = Absorbance of unknown
C = Calibration factor

Data Analysis

The data were analyzed for statistical significance between the controls and the Pb-exposed fish by the Student's t-test. Significant differences were establish at the 0.05 level.

RESULTS

Water Quality

The quality of water in the holding tanks for the experiment was within the acceptable range for fish production as shown in Table 1.

Table 1. Ranges of the water quality in the holding tanks.

Parameter		Holding tanks
Dissolved oxygen	(ppm)	6.9 - 8.3
Temperature	(°C)	27.3 - 28.0
pH		7.1 - 7.8
Ammonium	(ppm)	0.48 - 0.3
Total hardness	(ppm)	53.4 - 55.7

Lead Concentrations in the Exposure Tanks Water

Actual Pb concentrations in the experimental tanks were found to be higher than the nominal concentrations. The deviations from the expected values were less if the background Pb in control water was subtracted. All the subsequent results were compared to actual concentrations. The determined Pb concentrations were the total Pb levels since the water samples were stored at pH 1 with nitric acid until analysis. The Pb concentrations in the water (except for the control water) were found to have decreased after exposure periods of 48 hours, but the levels agreed well with the nominal levels. On the other hand, the Pb concentration of the control water tanks increased after exposure for 48 hours, but the cause of increasing level was unknown. Statistical significance between Pb concentrations at 0 and 48 hours using Student's t-test was not established at 5% level.

ALA-D Activity

After 4 weeks, the erythrocyte ALA-D activity in the Pb-exposed fish showed a successive decrease (by 24%, 41%, and 80%, respectively) with the increased Pb concentration in the water (Figure 1). No obvious recovery of ALA-D activity was seen after the fish were transferred to clean water for 7 weeks.

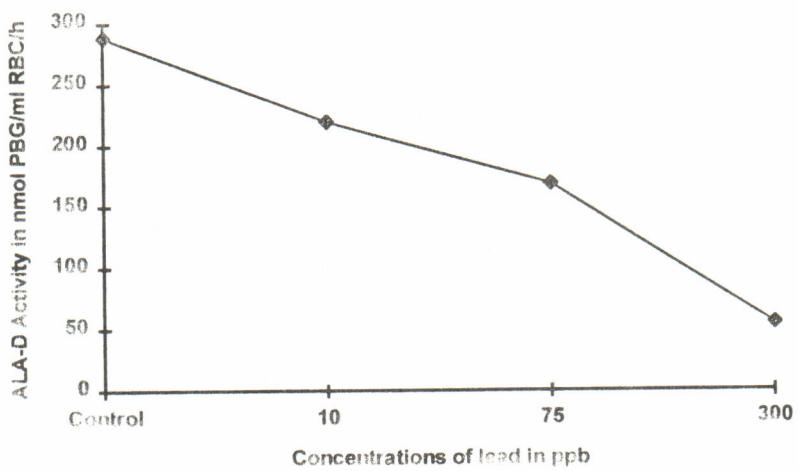

Figure 1. Variation in erythrocyte δ-ALA-D activity of *Puntius gonionotus* after 4 weeks of exposure.

Blood Glucose and Lactate Levels

Lead induced biochemical alterations included a hyperglycemic response at 4 weeks, when the blood glucose values for the highest Pb concentration exposure were 46.5% higher than the control values, but no significant differences was found between the other groups of 10 and 75ppb of lead concentrations. By contrast, a significant reduction occurred in the blood glucose level of Javanese carp exposed to 75ppb of Pb.

On the other hand, no significant deviation in lactate level with any of the concentrations of toxicant was detected. The highest level of lactate after exposure to Pb was found to be 57.06mg/100mL and for the lowest level of lactate was 35.76mg/100mL (Figure 2).

Total Hemoglobin

After 4 weeks, fish exposed to 10, 75 and 300 ppb showed no significant deviations from the control group. Fish exposed to 300 ppb showed reduced values of hemoglobin to 9.6±0.04g/100mL compared to a control group of 12.6±0.04g/100mL (Figure 3).

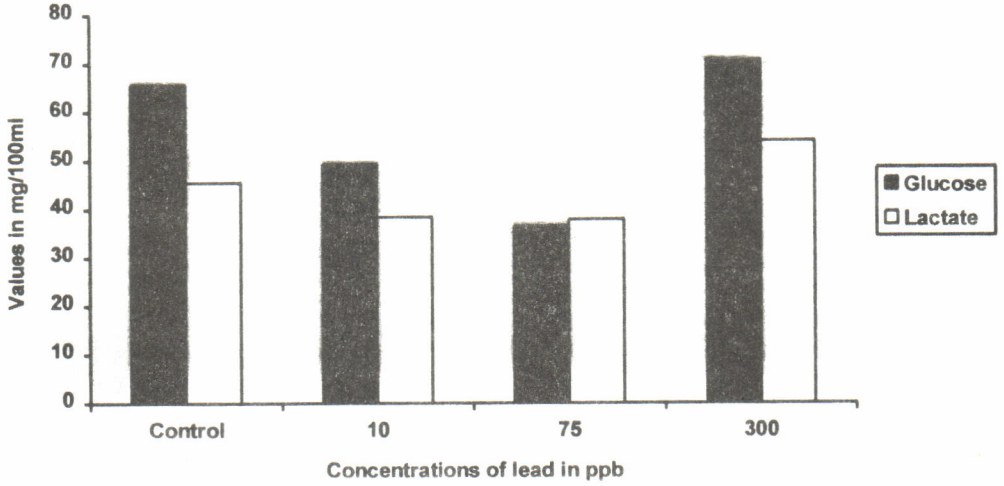

Figure 2. Blood glucose and lactate levels after 4 weeks of exposure of fish to different lead concentrations.

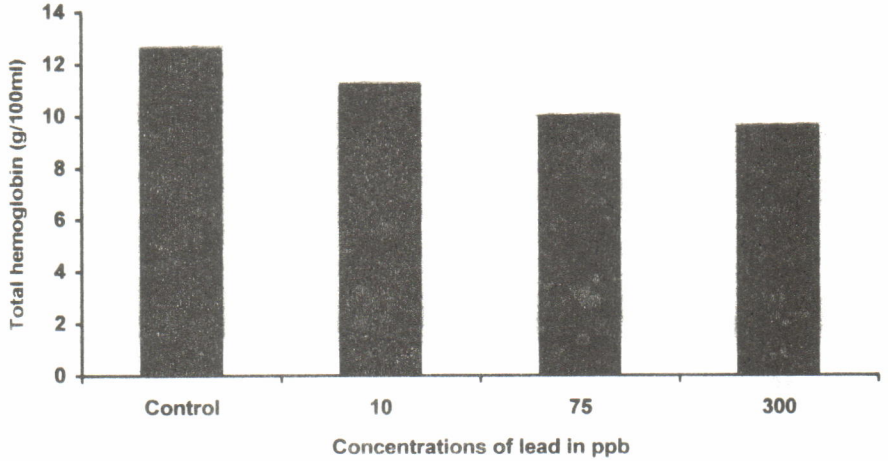

Figure 3. Hemoglobin levels after 4 weeks of exposure of fish to different lead concentrations

DISCUSSION

The results show that the ALA-D activity in the erythrocytes is strongly inhibited by inorganic Pb and that the degree of this inhibition increases with increased Pb concentration in the water. An inhibitory action of Pb on the erythrocyte ALA-D is well documented for various vertebrates including man (Botts, 1977). The primary mechanism behind this inhibition seems to be that essential sulphydryl groups in ALA-D are inactivated by Pb (Gibson and Goldberg, 1970, Granick *et al.*, 1973).

In Pb-exposed mammals, the erythrocytes' ALA-D activity shows a good negative correlation to the concentration of Pb in blood (Hernberg *et al.*, 1970). A similar correlation is apparently valid also for fish exposed to Pb (Hodson *et al.*, 1977; Nakagawa *et al.*, 1995). The last mentioned study also revealed a negative correlation between the erythrocytes' ALA-D activity and the logarithm of Pb concentration in the water. In the present study, it was shown that erythrocyte ALA-D activities of Javanese carp were depressed with increasing Pb concentration in water after exposure to Pb concentrations of 10, 75 and 300ppb. The degree of enzyme inhibition induced by Pb in various nominal concentrations in the present study. This agrees well with the results obtained by Hodson *et al.* (1977) and Nakagawa *et al.* (1995), that ALA-D in fish blood is a parameter that reflects the degree of Pb concentration.

After 4 weeks of exposure, there was a positive correlation between blood lead concentrations and water concentrations. A similar result was reported for common carp (Nakagawa *et al.*, 1995). Blood lead concentrations in Javanese carp exposed to Pb concentration of 300ppb did not reach equilibrium, but continued to increase throughout 4 weeks of exposure. Blood lead concentration also reflected the degree of Pb contamination of fish. Moreover it was found that there is a clear negative correlation between blood lead concentrations and ALA-D activities in Javanese carp. Similar results were observed in the exposed test using rainbow trout and brook trout (Hodson *et al.*, 1977), common carp (Nakagawa *et al.*, 1995) and the field survey of longear sunfish, *Lepomis megalotis* (Dwyer *et al.*, 1988). Because ALA-D activity is inversely related to blood Pb, which in turn reflects the degree of Pb contamination of the fish, Hodson *et al.* (1977) reported the blood ALA-D is also useful for diagnosing past exposure to Pb, even if Pb in the water at the time of analysis is of the usual background concentration.

No significant recovery of the ALA-D activity in erythrocytes was seen after transfer of the Pb-exposed fish to clean water for 7 weeks. This differs from previous findings by Hodson *et al.* (1977) on rainbow trout and Nakagawa *et al.* (1995) using common carp, exposed to Pb in freshwater is inconsistent might be explained by the shorter exposure period (2 weeks) in the latter studies compared to the present (4 weeks).

It is obvious from the present results that the periods of 7 weeks stay in Pb-free water is not sufficient for the erythrocyte ALA-D activity to return to normal levels. This slow recovery makes this enzyme useful as a diagnostic indicator for detecting Pb exposure fish, even in water, where the Pb content has returned to background levels. The present finding on the erythrocyte ALA-D also supports the opinion of Hodson (1976) and Hodson *et al.* (1977) that determination of ALA-D activity provides a sensitive and fast technique for determination of sublethal disturbances induced by Pb. The test can therefore be used for the estimation of Pb exposure of fish and hence, Pb in water. The measurement of this enzyme seems to be much easier for detecting Pb poisoning than the determination of Pb in water or in blood and other tissues (Morgan & Burch, 1972).

With the highest Pb concentration (300ppb), a hyperglycemic response was induced when blood glucose value was 46.5% higher than the control value. Similar results were observed with the blood lactate level (24%). Glucose and lactate increases are usual responses after

stress action (Soivio & Oikari, 1976), although in some cases, a decrease in glucose level may be due to disturbances on energetic pathways. Such a response has been detected in rainbow trout, *Salmo gairdneri* after exposed to 10, 75 and 300ppb Pb for 4 weeks (Haux *et al.*, 1986). Glucose is released by corticosteroids whose elevation has been described as a primary response to most stressors, including heavy metals (Donaldson, 1981). Lactate elevation could be related to anaerobic metabolism, which could be partially due to hypoxia generated by metal accumulation on the gills (Skidmore, 1970).

There was a slight decrease in blood hemoglobin concentration when exposed to the highest Pb concentrations, but still no significant deviations from the control group. In the present work, the decreased Hematocrit value after exposure to Pb contamination was indicative of a severe anaemic state causally related to prolonged Pb exposure. While the anaemia was evident only in fish exposed to the highest Pb concentration, the inhibition of erythrocyte ALA-D activity was apparent even in fish exposed to the two lower concentrations. Probably, the anaemia is largely a result of a disturbed hemoglobin synthesis due to the observed blocking effect of Pb on ALA-D. Other contributing action mechanisms are, however, possible. In mammals, it has been shown that the red cell survival time is shortened by Pb (Hernberg, 1976). The mechanisms by which Pb causes this effect are not fully understood, but changes such as increased osmotic resistance and increased mechanical fragility of the red blood cells are certainly involved (Waldron, 1966).

CONCLUSION

It can be concluded that Pb exposure causes a strong inhibition on enzyme ALA-D in fish, and that this effect probably contributes to the anaemic condition observed in fish exposed to the highest Pb concentration. The Pb-exposed fish showed a dose-dependant inhibition of erythrocyte ALA-D activity. The slow recovery of the ALA-D activity makes this enzyme useful as diagnostic indicator for detecting Pb exposure of fish, even several weeks after the exposure has ceased.

Despite the persistent, pronounced inhibition of ALA-D activity in the Pb-exposed fish, no significant effects could be detected on the hemoglobin content and Hematocrit values. Thus the consequence of the Pb-induced enzyme inhibition, in terms of erythrocyte function, is still an unanswered question.

Thus, similar to the situation in the highest vertebrates, the inhibition of ALA-D seems to be sensitive (perhaps the earliest) measurable effect of Pb exposure in fish. It is however difficult to evaluate the significance with respect to animal health, as most of the enzymes have a relatively large reserve capacity even in mammals (Hernberg, 1976). Further experiments are needed to elucidate the long-run consequences of the observed ALA-D inhibition on erythrocytes' function and ultimately on the well-being of the fish. Furthermore, the persistence of the enzyme inhibition must be a subject for further studies.

The Pb exposure also affected the carbohydrate metabolism. A persistent hypoglycemia and an initial slight increase in blood lactate were found after Pb exposure. The hypoglycemic response might be a result of Pb-induced morphological and functional changes in renal tubular cells. Such a renal effect is common in Pb-exposed mammals and is accompanied by glucosuria.

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