Histopathological biomarkers in juvenile silver catfish (Rhamdia quelen) exposed to a sublethal lead concentration

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ABSTRACT

The aim of this study was to determine the 96-h lethal concentration (96-h LC50) of lead (Pb) in silver catfish, Rhamdia quelen, and to determine histopathological biomarkers in fish exposed for 96-h to a sublethal concentration at 25% of the LC50. The 96-h LC50 was 108 mg/L. In gills, the length and thickness of lamella and thickness of the filament epithelium were significantly higher in fish exposed to Pb for 48-h than in control fish whereas the interlamellar distance decreased. In the liver, the area occupied by lipid droplets and size of hepatocytes showed significantly higher values after 24-h of exposure. The percentage of abnormal renal tubules was higher in fish exposed to Pb, exhibiting a time-dependent increase. These variations in histopathological biomarkers permit the definition of the overall response of R. quelen to Pb and the potential usefulness in the monitoring of Pb contamination.

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1. Introduction

Lead (Pb) contamination results primarily from air pollution by motor vehicles, industrial waste and non-recycled batteries (Mattos et al., 2009). Although the use of Pb by many industries has declined considerably, many rivers still show high levels of contamination, especially those near Pb mines, smelters or industries (Salibian et al., 2006). As has been reported by many authors, contaminated fish are considered a significant source of Pb poisoning in humans (Needleman, 2004).

In fish, responses to water contamination range from molecular alterations to structural changes in tissue architecture. A biomarker is an indicator of stress at different levels of biological organization and is used to evaluate the effects of different contaminants in organisms (Lam and Gray, 2003). In particular, histopathological biomarkers have some advantages over biochemical or physiological biomarkers such as ease of sample collection and storage, the study of fish that preclude dissection due to their small size and the possibility to assess specific sites of cellular or tissue injuries (Handy et al., 2002). Fish are generally considered one of the most feasible indicators of heavy metal contamination in aquatic ecosystems because of their different trophic levels, sensibility to water toxicants and widespread distribution (Alibabici et al., 2007). The use of biomarkers in fish has been extensively studied for many years and it is considered a useful method in the monitoring and early detection of water contamination (Bernet et al., 1999; Stentiford et al., 2003).

The silver catfish or South American catfish (Rhamdia quelen, Quoy and Gaimard), is a freshwater fish widely distributed from Mexico to Argentina (Silfvergrip, 1996). Its omnivorous feeding habits and preference for eating benthic prey (Gomes et al., 2000) are important features in order to assess toxicants that accumulate in bottom sediment such as Pb (Demayo et al., 1982). In addition, R. quelen has greater resistance to adverse aquatic conditions than other species (Braun et al., 2006), and thus it is a species of choice for the detection of early water contamination by means of histopathological biomarkers. Therefore, the aim of this study was to determine the 96-h lethal concentration 50 (96-h LC50) of Pb in R. quelen and to determine histopathological biomarkers and the potential usefulness of the R. quelen in the assessment of Pb water contamination.

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2. Materials and methods

The methodology of this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process no. 23081.008430/2007-05).

2.1. Fish

Juveniles of R. quelen (5–20 g) were bought from a fish culture in Santa Maria (Southern Brazil). They were held in continuously aerated water in 250 l tanks for 7 days prior to the beginning of the experiments in the facilities of the Fish Physiology Laboratory at the Universidade Federal de Santa Maria. Fish were fed to satiety with commercial pellet (32% crude protein, Supra Juvenil, Carazinho, Brazil) every 24-h until the day before the experiment.

2.2. Determination of 96-h lethal concentration

Since no references on the sensitivity of silver catfish to Pb were available, before conducting the final test to determine the lethal concentration (LC50), a preliminary series of 96-h exposure to 25, 50, 100, 150 and 200 mg l⁻¹ Pb [as Pb(NO₃)₂] were performed in five different aquariums containing five fish each. All fish exposed to 200 mg l⁻¹ Pb died whereas the survival rate in all other concentrations was 100%. Thus, the final test consisted of four groups of five fish each exposed to concentrations of 160, 170, 180 and 190 mg of Pb per l of water and a control group with no Pb exposure. Three replications were carried out for each concentration, and mortality was recorded daily for four days. Since Pb added to water has an insoluble fraction (Demayo et al., 1982), the effective waterborne Pb concentration was determined by graphite furnace atomic absorption spectrometry (GF AAS) or inductively coupled plasma mass spectrometry (ICP-MS). The LC50 value was estimated by the Trimmed Spearman–Karber method (TSK) using free software (U.S. EPA, 1990).

2.3. Sublethal exposure

In order to evaluate Pb effects on organ structure, fish were randomly distributed in two groups. The treatment group (n = 32) was exposed to a sublethal Pb concentration of 27 mg l⁻¹ Pb corresponding to 25% of LC50 whereas the control group (n = 10) was composed of fish unexposed to Pb. Water parameters during the test were as follows: temperature 19 °C, pH 6.5, hardness 20 mg l⁻¹ CaCO₃, alkalinity 10 ± 2.5 mg l⁻¹ CaCO₃, total ammonia 0.34 ± 0.15 mg l⁻¹, nitrite below 0.05 mg l⁻¹ and dissolved oxygen above 6 mg l⁻¹. The experiment was conducted for 96-h considering as time zero the moment when fish were exposed to Pb. Before sampling, fish were sedated with 70 mg l⁻¹ eugenol and euthanized by sectioning the spinal cord. Samples of gills, liver, spleen and kidney were dissected out at 24, 48, 72 and 96 h of exposure (he) and cut into small pieces.

2.4. Histopathology and morphometry

Samples were placed in Bouin’s fluid for 12 h, transferred to ethanol 70%, dehydrated in an increasing ethanol series and embedded in paraffin wax. Tissue blocks were sectioned at 3–5 μm thick and stained with haematoxylin–eosin. Finally, images were obtained using a Leica DM 750 photomicroscope equipped with a Leica EC3 camera. For each fish sampled and organ studied, three digitized images were collected using the 40 × objective. Different variables (Table 1) were assessed using image analyzes software, Image J v. 1.45s (in the public domain and available from the National Institutes of Health, USA).

2.5. Statistical analyzes

For each variable analyzed, differences among groups (p < 0.05) were tested for significance by one-way ANOVA and Bonferroni multiple comparison tests. Comparisons were made among fish exposed to Pb for different time periods and also with control group. All tests were done using JMP Software Version 5.1.1 (SAS Institute Inc.).

3. Results

3.1. Determination of 96-h LC50

The effective waterborne Pb concentrations determined by GF AAS or ICP-MS were 98.7, 105, 111 and 117 mg l⁻¹ for dilutions of 10², 10¹, 10⁰ and 10⁻¹ of Pb, respectively.

The LC50 determined by the TSK method from data obtained from effective concentrations of Pb was 108 mg l⁻¹. After 96-he, 100% survival was observed in control fish as well as in fish exposed to the lowest Pb concentration. Fish exposed to 105 mg l⁻¹ and 111 mg l⁻¹ Pb exhibited 75% and 25% survival rates at 96-he, respectively. When fish were immersed at the highest Pb concentration, all fish died after 72-he (Fig. 1).

3.2. Histopathology and morphometry

The structure of gills (Fig. 2a), kidney (Fig. 4a), spleen (Fig. 4c) and liver (Fig. 4e), sampled in control fish was in accordance with previous reports in teleosts and are shown in order to allow the comparative assessment of lesions observed in fish exposed to Pb.

3.2.1. Gills

The most important changes observed after 24-he were lamellar edema, epithelial lifting (Fig. 2b) and epithelial necrosis (Fig. 2b, inset), the latter being the most common lesion. However, these alterations decreased from 24-he onwards. Contrarily, cellular hypertrophy, epithelial hyperplasia (Fig. 2c) and lamellar fusion (Fig. 2d) increased in a time-dependent manner. The length of

<table>
<thead>
<tr>
<th>Organ</th>
<th>Variable name</th>
<th>Definition</th>
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<tr>
<td>Gills</td>
<td>Length of lamella</td>
<td>Length of lamella from base to free tip (μm)</td>
</tr>
<tr>
<td></td>
<td>Thickness of lamella</td>
<td>Thickness of lamella at the base (μm)</td>
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<tr>
<td></td>
<td>Interalamellar distance</td>
<td>Distance between the bases of two adjacent lamellae (μm)</td>
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<td></td>
<td>Thickness of the epithelium of the filaments</td>
<td>Thickness of the epithelium of gill filaments (μm)</td>
</tr>
<tr>
<td>Liver</td>
<td>Area of lipid droplets in hepatocytes</td>
<td>Percentage of area occupied by lipid droplets in the cytoplasm of hepatocytes (%)</td>
</tr>
<tr>
<td></td>
<td>Size of hepatocytes</td>
<td>Mean size of hepatocytes with the nucleus sectioned (μm²)</td>
</tr>
<tr>
<td></td>
<td>Total number of nuclei</td>
<td>Quantity of hepatocytes nuclei assessed in an area of 64,000 μm²</td>
</tr>
<tr>
<td>Kidney</td>
<td>Percentage of abnormal renal tubules</td>
<td>Percentage of renal tubules with lesions such as degeneration, epithelial desquamation or necrosis (%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>Area occupied by lymphoid tissue</td>
<td>Percentage of area occupied by lymphoid tissue in a total spleen area of 64,000 μm² (%)</td>
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</table>
lamella was significantly longer in exposed fish compared with unexposed fish, showing the highest value at 48-he. At 72-he and 96-he, length of lamella decreased but still remained higher than the control group (Fig. 3a). The thickness of lamella increased progressively from 24-he to 96-he exhibiting significantly higher values than in unexposed fish from 48-he onwards (Fig. 3b). The interlamellar distance decreased significantly from 48-he onwards in comparison with the control group (Fig. 3c). The thickness of the filament epithelium showed a significant difference with the control group only at 48-he (Fig. 3d).

3.2.2. Kidney

In kidneys, cloudy swelling of epithelial tubular cells was the first lesion observed at 24-he while necrosis of the tubular cells with presence of desquamated cells in the collector tubules was observed in latter samplings (Fig. 4b). The severity and extension of the lesions increased in relation to the hours exposed since the percentage of abnormal renal tubules rose in a time-dependent manner (Fig. 3e). No remarkable lesions were observed in kidney haemolymphopoietic tissue. Free melanomacrophages and melanomacrophages centers in this organ did not vary in number or size between control and Pb-treated fish.

3.2.3. Spleen

In the spleen of fish exposed to Pb, lymphatic depletion around blood vessels and an increased number of lymphocytes within splenic sinusoids and ellipsoids were observed (Fig. 4d). However, the percentage of area occupied by lymphoid tissue in fish exposed to Pb did not show significant differences in comparison with the control group due to the relocation of lymphocytes. Fish exposed for 48-h exhibited a significantly lower value of the percentage of area occupied by lymphoid tissue than fish sampled at the end of the experiment (Fig. 3f).

3.2.4. Liver

The liver of fish exposed to Pb showed venous congestion, moderate vacuolization and some areas of necrotic tissue (Fig. 4f). Among the variables analyzed in this organ, the area of lipid droplets in the hepatocytes was significantly higher after 24-he than in the control fish but decreased at 48-he. From 72-he, values returned to similar control levels (Fig. 5a). The size of the hepatocytes showed a significant increase at 24-he and then decreased from 48-he onwards (Fig. 5b). The number of hepatocytes nuclei did not show significant differences throughout the test (Fig. 5c).
4. Discussion

In fish, Pb LC50 is the result of a complex interaction among water hardness, pH, previous metal exposure, fish age and species sensitivity (Demayo et al., 1982). Therefore, a wide variation in LC50 among fishes can be expected. Lead 96-h LC50 was 925 mg l\(^{-1}\) (water hardness 23.4 mg l\(^{-1}\)) for *Heteropneustes fossilis* (Bloch) (Parashar and Banerjee, 2002), 2.15 mg l\(^{-1}\) for *Oreochromis niloticus* (Linnaeus) 1.72 mg l\(^{-1}\) for *Clarias gariepinus* (Burchell) (both water hardness 4.5–7.6 mg l\(^{-1}\)) (Oladimeji and Offem, 1989) and 95 mg l\(^{-1}\) for *Prochilodus lineatus* (Valenciennes) (water hardness 82 mg l\(^{-1}\)) (Martinez et al., 2004). In the present study, the 96-h LC50 was 108 mg l\(^{-1}\) (water hardness 20 mg l\(^{-1}\)) thereby demonstrating that *R. quelen* is one of the most resistant fish to Pb exposure. This is considered to be an advantage in order to establish this species as a useful Pb pollution bioindicator. As was reported for fish toxicity to other metals, such as copper and zinc (Ebrahimpour et al., 2010), water hardness has an effect on Pb LC50 due to the fact that metal toxicity decreases with the increase of water hardness (Demayo et al., 1982). However, the results of LC50 in different fish species under different water hardness conditions show that the relationship between water hardness and Pb toxicity is not constant across species since many other factors could influence the LC50 determination (Demayo et al., 1982). With these factors in mind, LC50 determination must be ascertained for each fish species under controlled conditions.

Most of the target organs for heavy metals toxicity are those involved in uptake, detoxification and excretion (Roesijadi, 1992), i.e., the gills, liver and kidneys. In addition, the spleen can also be affected as it takes part in blood filtration (Press and Evensen, 1999), and thus it might be exposed to circulating heavy metals.

Gills play an important role in gas exchange, osmoregulation and maintenance of acid-base balance. Gills are a frequent location of histopathological changes induced by heavy metal exposure due

![Fig. 3](image-url). Mean ± SEM values of variables assessed in gills (a, b, c, d), kidney (e) and spleen (f) of control and Pb-exposed fish for 96-h. Different letters indicate significant differences among groups (ANOVA, Bonferroni post-hoc test, \(P < 0.05\)).
to the direct interaction with the aquatic environment, constant ionic exchange and their delicate structure (Mallatt, 1985). In line with these findings, many of the alterations observed in gills are common in several fish species. In *H. fossilis*, it was observed that the main lesion was epithelial lifting and cell damage with posterior hyperplasia and lamellar fusion after a 96-h Pb exposure (Parashar and Banerjee, 2002). Similar changes were described in *Tinca tinca* (Roncero et al., 1990). In *P. lineatus* exposed to Pb for 96-h, the commonest lesions observed were epithelial lifting, hyperplasia and lamellar aneurism (Martinez et al., 2004). The latter was considered by the authors as the most specific for heavy metal toxicity. The lesions induced by acute Pb toxicity in *R. quelen* were similar to those described in the above mentioned species. During the first hours of exposure, degenerative changes such as epithelial lifting and necrosis prevailed whereas proliferative tissue reactions like cellular hypertrophy and epithelial hyperplasia that produced lamellar fusion predominated from 48-h onwards. However, contrary to what was previously described by Martinez et al. (2004) in *P. lineatus*, lamellar aneurisms were not observed in *R. quelen* exposed to Pb. In our opinion, this lesion reported in *P. lineatus* was due to the sacrificing method employed, and it was not related to Pb exposure since the use of physical euthanizing methods.

Fig. 4. (a) Normal renal tissue in unexposed fish. Different types of renal tubules (arrowheads) and the haemolymphopoietic tissue (asterisks) are shown. Bar = 50 μm; (b) Renal tubules with cloudy swelling of their epithelial cells (arrowheads) as well as epithelial desquamation within the tubules (arrows) are seen after 48-h. Bar = 50 μm; (c) Spleen architecture in unexposed fish. Lymphoid cell populations (dotted area) associated to splenic ellipsoids (arrowheads). Splenic sinusoids (asterisks) are also seen. Bar = 50 μm; (d) Lymphatic depletion around two splenic ellipsoids (arrowheads) after 72-h exposure. Note the presence of lymphocytes filling the vascular lumen of ellipsoids. Bar = 50 μm; (e) Normal liver structure in unexposed fish. High lipid vacuolation in hepatocytes is clearly seen. Bar = 50 μm; (f) Areas of non-vacuolated hepatocytes (arrowheads) interspersed with vacuolated hepatocytes (arrows) in liver of fish exposed to lead for 96-h. Bar = 50 μm. Inset: Venous congestion (asterisk) with necrotic tissue around the central vein (arrowheads). Bar = 50 μm.
methods with no previous sedation can cause iatrogenic gill telangiectasia in fish (Noga, 2010).

The morphometric changes observed in the gills of *R. quelen* were mainly a significant increase in the thickness of lamellar and filament epithelia with a consequent reduction of the interlamellar distance after 48-h. These proliferative tissue responses might be triggered to reduce the local toxic effect of Pb. Nevertheless, these structural modifications increase the diffusion distance, and thus gas exchange could be reduced. In line with this, a significant increase in the length of lamella during the first 48-h of Pb exposure was observed. This can be interpreted as a compensatory mechanism to increase the surface for gas exchange in order to minimize the effects of epithelium proliferation. Although the lesions induced by Pb exposure reported in other fish species (Martínez et al., 2004; Parashar and Banerjee, 2002; Roncero et al., 1990) are similar to those described in *R. quelen*, to our knowledge this is the first study to quantify several morphometric variables in the gills of fish exposed to an acute sublethal Pb concentration.

As stated above, kidneys are an important organ involved in detoxification and therefore are a target organ for heavy metals toxicity (Roesijadi, 1992). Degeneration, epithelial desquamation and necrosis of tubular cells were the most frequent lesions observed in *R. quelen* exposed to Pb. The extension of lesions increased in a time-dependent manner as was demonstrated by the significant rise in the percentage of abnormal renal tubules among samplings. In other fish species exposed to lower Pb concentrations for longer time, such as *Sarotherodon galilaeus* (Linnaeus) and *Chelon parisa* (Hamilton), similar histopathological changes like tubular epithelial degeneration, necrosis and elimination of the epithelia from the distal tubule have been described (Al-Zahaby et al., 1998; Pandey et al., 1997).

As in other teleosts (Press and Evensen, 1999), the spleen in control *R. quelen* showed a red pulp mainly constituted of sinusoids and ellipsoids containing different cellular types, such as erythrocytes and macrophages, and a white pulp composed mainly of lymphoid cells arranged around melanomacrophages centers and blood vessels. Few studies have assessed the effects of Pb on the immune system. Dunier (1996) described a Pb-induced in vitro lymphocyte proliferation in *Cyprinus carpio* (Linnaeus). Similar results were observed in vivo in *Anguilla anguilla* (Linnaeus) with an increase of circulating lymphocytes in fish exposed to Pb for 30 days (Santos and Hall, 1990). Shah and Altindag (2005) reported that *T. tinca* exposed to 25% of LC50 Pb for 96-h exhibited a significant two-fold increase of white blood cell count in comparison with control fish. The authors suggest that this situation could be attributed to an efflux of lymphocytes from lymphopoietic loci, i.e., kidneys and spleen. This is in agreement with what was observed in *R. quelen* since a relocation of lymphocytes from the area surrounding the blood vessels to the vascular lumen was clearly evidenced. However, in this study, there were no differences in the percentage of the area occupied by lymphoid tissue between the control group and the group exposed to Pb. This variable only exhibited a significantly lower value in fish exposed to Pb for 48-h in comparison with fish sampled at the end of the experiment. This fact could be related to an early transfer of lymphocytes from spleen to the general circulation as other authors have previously suggested (Shah and Altindag, 2005).

Despite the role of liver in heavy metal detoxification (Heath, 1995), few studies have described the effects of acute Pb toxicity on liver histopathology in fish. As was observed in *R. quelen*, Suiçmez et al. (2006) reported venous congestion and hepatocytes necrosis in *Onchorhynchus mykiss* (Walbaum) exposed to 10 mg l\(^{-1}\) Pb for 72-h. After 7 days of exposure to the same concentration, Khdir et al. (2012) only observed an increase of hepatocyte vacuolization in *O. niloticus*.

Vacuolization of hepatocytes as a consequence of the response of this cell type to toxicants is associated with the inhibition of protein synthesis, energy depletion, disaggregation of mitochondria or shifts in substrate utilization (Hinton and Laurén, 1990). This change was registered in *R. quelen*, and it was associated with a significantly higher value of the area of lipid droplets which also led to a significant increase in the size of hepatocyte after 24-h to Pb. From 48-h onwards, both variables returned to levels similar to control levels. Oljjo et al. (2005) also observed hepatocyte hypertrophy in *C. gariepinus* exposed to a lower Pb concentration (0.08 mg l\(^{-1}\)) but for a longer exposure time (18 days).

The total number of nuclei registered in *R. quelen* in order to assess hepatocytes hyperplasia did not show significant changes between control and Pb-treated fish, which suggests that cellular proliferation was not a tissue response to Pb injury in the liver.

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**Fig. 5.** Mean ± SEM values of variables assessed in liver of control and lead exposed fish at different times. Different letters indicate significant differences among groups (ANOVA, Bonferroni post-hoc test, *P* < 0.05).

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5. Conclusions

The present study determines the Pb 96-h LC50 in *R. quelen*. Although most lesions described in this species were similar to those reported in other teleosts exposed to Pb, this is the first study that describes the lesions and quantifies by means of morphometry several variables which define the overall multigorgan response to acute sublethal Pb toxicity in fish. These tissue reactions were characterized by three facts. First, after 24-h of exposure, there was an increase of the area occupied by lipid droplets and the size of hepatocytes. Second, in the first 48-h, there was an increase in the length and thickness of lamella and thickness of the filament epithelium of the gills. Lastly, from the beginning to the end of the experiment, there was a time-dependent increase in the percentage of abnormal renal tubules. In addition, the omnivorous feeding habits and preference for eating benthic prey make *R. quelen* an ideal species for the assessment of Pb water contamination through the use of histopathological biomarkers.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2014.11.036.

References


