In situ and laboratory evaluation of toxicity with *Danio rerio* Buchanan (1822) and *Poecilia reticulata* Peters (1859)

Avaliação in situ e em laboratório da toxicidade com *Danio rerio* Buchanan (1822) e *Poecilia reticulata* Peters (1859)

Fracácio, R.[1], Rodriguês, BK.[1], Campagna, AF.[1], Verani, NF.[1],

Dornfeld, CB.[2] and Espíndola, ELG.[2]

[1]Departamento de Hidrobiologia – DHB, Universidade Federal de São Carlos – UFSCar,
Rod. Washington Luis, SP-310, Km 235, CEP 13565-905, São Carlos, SP, Brazil
e-mail: rfracacio@uol.com.br, beatriz_kr@yahoo.com.br, aline.campagna@gmail.com, dnfv@power.ufscar.br

[2]Departamento de Hidráulica e Saneamento, Universidade de São Paulo – USP,
Av. Trabalhador São Carlense, 400, CEP 13560-600, São Carlos, SP, Brazil
e-mail: carol_buso@yahoo.com.br, elgaeta@sc.usp.br,

Abstract: Aim: The objective of this study was to evaluate the toxicity of various water pollutants in a lotic environment, the Monjolinho River (São Carlos, SP, Brazil). **Methods:** by means of in situ (96 hours) and laboratory (7 days) tests using adults and juveniles of *Danio rerio* and *Poecilia reticulata*. **Results:** The responses were observed in terms of mortality and changes in gill tissues. There was greater mortality in the in situ than in the laboratory tests. In both tests the *P. reticulata* juveniles were more sensitive than the other phases of the species used. The histological analyses of the surviving fish of both species showed that the harmful effects detected on the gills were caused by the various pollutants found, such as the metals detected in the water and sediment (Cd, Cu^{++}, Cr, Zn, Mn and Fe), and the organochlorides available in the water (aldrin, heptachlor and endosulfan sulphate). The concentrations of these pollutants were above the limits established for aquatic life protection (CONAMA 357/05). **Conclusions:** high levels of pollution in Monjolinho River.

Keywords: toxicity tests, experiments in situ, *Danio rerio, Poecilia reticulata*, histology, lotic environment.

Resumo: Objetivo: O objetivo desta pesquisa foi avaliar a toxicidade da água de um ambiente lótico, o rio Monjolinho (São Carlos, SP, Brasil). **Métodos:** por meio de ensaios in situ (96 horas) e laboratoriais (7 dias), utilizando-se adultos e juvenis de *Danio rerio* e de *Poecilia reticulata*. **Resultados:** Foram observadas as respostas em termos da mortalidade e das alterações teciduais de brânquias. Os ensaios in situ, revelaram maior mortalidade do que os ensaios em laboratório. Tanto em in situ como em laboratório os juvenis de *P. reticulata* foram mais sensíveis do que as demais fases das espécies utilizadas. As análises histológicas dos peixes sobreviventes, de ambas as espécies, apontaram que os efeitos deletérios nas brânquias foram, de modo geral, causados pelos diversos poluentes detectados como alguns metais na água e metais biodisponíveis no sedimento (Cd, Cu^{++}, Cr, Zn, Mn and Fe) e organoclorados avaliados na água (aldrin, heptacloro e endosulfan sulfato), os quais apresentaram-se acima dos limites estabelecidos para a proteção da vida aquática (CONAMA 357/05). **Conclusões:** elevado grau de poluição instalada no rio Monjolinho.

Palavras-chave: testes de toxicidade, experimentos in situ, *Danio rerio*, *Poecilia reticulata*, histologia, ambiente lótico.

1. Introduction

Human activities in river basins produce pollution residues in the water, compromising multiple uses (Lloyd, 1992; Tucker and Burton, 1999; Tundisi, 2001; Torre et al., 2002). Chemical analyses can detect and quantify these pollutants but cannot evaluate their effects on the biota. It is necessary to conduct toxicity tests, by exposure of organisms of interest either in the laboratory or in the field, to evaluate their responses, such as mortality, stunted growth and reproduction and biochemical, physiological and histological changes complementing studies of the quality of water bodies (Hoffman, 2002; Matsui, 2002). In situ toxicity tests allow evaluation of the responses of the organisms under conditions as near as possible to those in nature, reflecting the various interactions of the pollutants. Interactions are influenced by the daily fluctuations of temperature, oxygen, turbidity and pH resulting in changes in the toxic conditions of the environment (Chappie and Burton, 2000; Ringwood et al., 2002). In laboratory tests, the variables and other conditions can be more closely controlled, however there is a loss of information because the responses only partially reflect the real environmental conditions (Forstner, 1990). Various in situ toxicity tests have been developed to monitor the toxic effects of aquatic environments, with organisms of different trophic levels (Meregalli et al., 2000). In Brazil, this approach is still not widely used (Dornfeld, et al., 2006). The objective of this study was to evaluate the water toxicity in a lotic environment (Monjolinho River, São Carlos, São Paulo, Brazil), by carrying out in situ (96 hours) and laboratory (7 days) tests, using adults and juveniles of two freshwater fish species, *Danio rerio* and *Poecilia reticulata.*

2. Material and Methods

2.1. Study area and sampling stations

The watershed of the Monjolinho River covers about 275 km^2, most of it within the municipality of São Carlos in the state of São Paulo, Brazil. The river receives waters from several tributaries and runs in an artificial channel through the urban area of São Carlos. Due to human activity, it receives industrial wastes (tannery compounds, paper, paints, foodstuffs and others) and raw sewage. After the urban stretch, the river runs through areas with stock raising and farming, mainly sugarcane, before flowing into the Jacaré-Guaçu River (Figure 1). The water from this river supplies 20% of the needs of the city of São Carlos and also is used for irrigation of truck farms (Espíndola, 2000). Samples were collected in July/03 (dry season) and April/04 (wet season), at four sampling sites, considering previous studies (Sé, 1992; Guereschi, 1995; Mendes, 1998; Barreto, 1999; Peláez-Rodrigues, 2000; Fracácio et al., 2000 and Marinelli et al., 2000). These sites were: A (Headwaters 22° 00' 33.0" S and 47° 50' 07.9" WO) – scanty native vegetation and extensive cultivation and stock breeding (July/03 and April/04); B (Fallen Bridge 22° 02' 04.3" S and 47° 57' 25.7" WO) – characterized by inflow of domestic and industrial wastes from the urban area of São Carlos (July/03), which was replaced by another sample station, at the start of the urban area, Point C (UFSCar 21° 59' 25.2" S and 47° 53' 29.4" WO) in April/04; and D (Confluence 22° 03' 32.2" S and 48° 05' 22.8" WO) – a rural area with extensive sugarcane fields, receiving all the residues generated by the municipality. We set up the in situ tests at sites A, B or C and D in each corresponding period (Figure 1). For the limnological and sediment analyses and laboratory tests, we collected samples at the four stations in July/03 and April/04.

2.2. Water sampling

We collected the samples daily in plastic bottles (0.5 L) for analysis of total phosphorus (TP) and total organic nitrogen (TON) (APHA, 1995). For analysis of total metals (Cd, Cu^{++}, Cr, Zn, Mn and Fe), we used larger plastic bottles (1 L), and fixed the samples in the field with 1.5 mL of concentrated nitric acid (APHA, 1995). We measured the temperature, dissolved oxygen, conductivity and pH in the field with a Horiba-U10 portable multiparameter water quality meter. For the toxicity tests, we collected the samples in plastic jugs (5 L) and kept them in a refrigerator for 24 hours until conducting the tests.

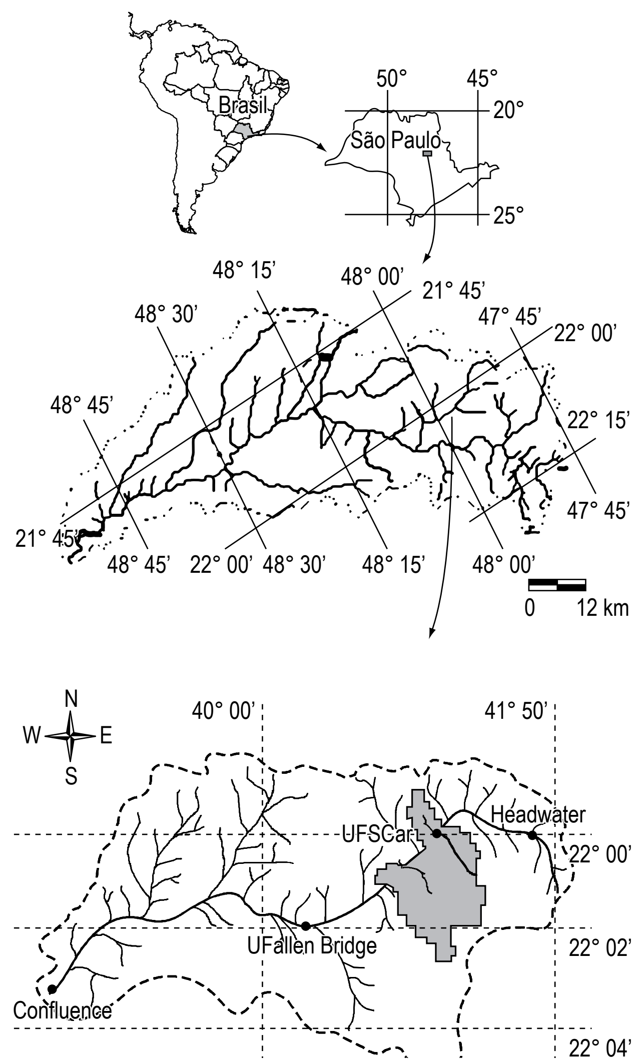

Figure 1. Study area in the state of São Paulo, Brazil: the location of sampling stations. The gray area is the urban part of São Carlos.

2.3. Sediment sampling

We collected the sediments with an Eckman-Birge dredge, with three repetitions at each site. The samples were homogenized and placed in plastic recipients (1.5 kg), and kept at room temperature for drying and subsequent analysis of bioavailable metals (Rosa and Azcue, 1997) and granulometry (ABNT, 1968).

2.4. Sensitivity tests

The *D. rerio* and *P. reticulata* adults and juveniles were obtained commercially and kept in the laboratory, in 25-L aquariums, with daily renewal of 1/3 of the cultivation water with reconstituted water (ABNT, 2003). After a five-day acclimation period, a static sensitivity test was carried out with potassium dichromate for a period of 96 hours for *D. rerio* and 48 hours for *P. reticulata*. The sensitivity tests indicated that the lots of fish were of good quality, permitting their use in the toxicity tests.

2.5. Initial biometry of the test organisms

We selected 20 individuals from each of four lots obtained commercially, to measure the standard length (with a Mitutoyo pachymeter) and the fresh weight (in grams on a Scientech AS 210 scale). We then calculated the variation of coefficient to evaluate the homogeneity of individuals (Ayres, 2000). When homogeneity was not observed, the lot was substituted.

2.6. Laboratory tests with the water samples

The chronic toxicity tests with the water samples followed the methods described in SEMA (1988) and CETESB (1994) for *D. rerio* larvae, adapted for juvenile and adult organisms of *D. rerio* and *P. reticulata.* We ran two replicas of each test with ten fish each, including the control. The test recipients were made of clear plastic sacks supported by rigid plastic frames. For the adult fish, we used recipients with capacity of 2 L, in which 1.5 L of sample water was added. For the juvenile fish the capacity was 1 L, with 800 mL of sample water, in both cases to maintain a ratio of 1 g of fish per liter of water. The tests lasted seven days, under static conditions, with controlled photoperiod, water temperature and oxygenation (12:12-light/dark; 23 ± 1 °C and constant oxygenation). We measured the pH, conductivity and hardness at the start, after 96 hours and at the end of the experiments, and recorded the fish mortality every 24 hours, removing the dead fish.

2.7. In situ toxicity tests

The fish were acclimated in the laboratory for five days in reconstituted water in aquariums with capacity of 250 L (ABNT, 2003). We then took the fish to the field in Styrofoam containers (to prevent temperature variations) holding plastic sacks containing ten fish each, in reconstituted water with sufficient oxygen to ensure their survival during transportation to the Monjolinho River. For acclimation, the sacks were immersed in the river water for 20 minutes. The holding chambers had been installed in the river 24 hours beforehand, to allow the dilution of possible residues contained in the materials used to make them. The chambers were made of PVC tubes with 7.5 cm diameter, 20 cm in height and capacity for 900 mL of water, closed at the top and bottom and with side cutouts. These openings were then covered with 300 μm mesh nylon netting, secured with nontoxic glue. Three of these chambers were placed in a vertical position, attached to a wood stake driven into the river bed, with their bases resting on the river bed (Figure 2). The fish species at the two phases were placed in each set of chambers (10 individuals/chamber), with three replicas, at each sampling station. In April/04 we used only two replicas because fewer organisms were available. The experiments lasted 96 hours, with evaluation of mortality at the end. The pH, conductivity, dissolved oxygen and water temperature were measured at the start and end of the tests with a portable Horiba-U10 meter.

2.8. Toxicity evaluation

To evaluate the significance of the mortality in the laboratory and in situ tests, we compared the ratio between the dead and living fish in the control group with the same measure at each of the sample sites, using the Fisher Exact Test (Ayres, 2000).

2.9. Histological evaluation of the gills

The surviving fish from the laboratory and in situ tests were sacrificed and fixed in Bouin's fluid for 24 hours, then washed in running water for the same period, placed in alcohol at 70% and subsequently dehydrated and diaphanized for imbedding in paraffin. Cross sections of 4 to 6 μm were then prepared with a steel-blade microtome (Micron HM 340E) and dyed with hematoxilin-eosin (HE). We evaluated the gill tissues under a light microscope, classifying the alterations according to Poleksic and Mitrovic-Tutundizc (1994).

3. Results and Discussion

3.1. Limnological analyses

According to Brazilian legislation, water bodies are classified as special, 1, 2, 3 and 4, according to the quality required for their preponderant use (CONAMA Resolution

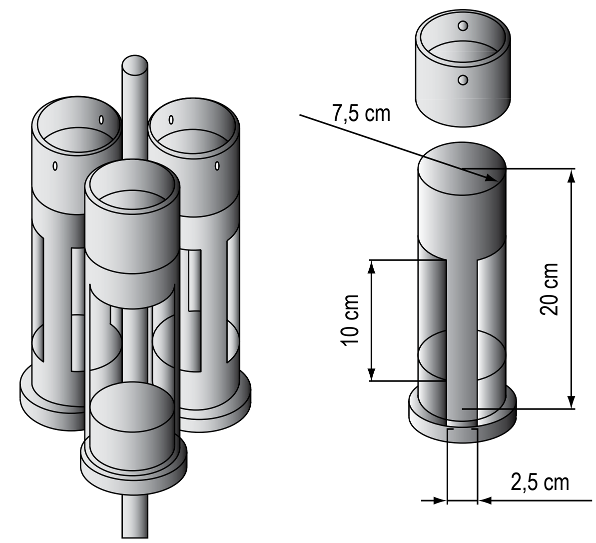

Figure 2. Holding chambers used in the in situ toxicity tests in the Monjolinho River.

357/2005). Each class has maximum limits established for physical and chemical variables, except class 4, which is for navigation and esthetic harmony, without restrictions on pollutants. The Monjolinho River, at the last two sampling sites (Headwaters and UFSCar), falls in class 2, defined as for protection of aquatic communities, supply for human consumption after conventional treatment, primary contact recreation, irrigation and fish farming. The first two sampling stations (Fallen Bridge and Confluence) are in class 4. Analyzing the chrome, cadmium, copper, zinc, iron and manganese in the water, we found that all were present in at least one of the study periods. In the dry period (July/03), the metal concentrations were highest, except for cadmium at Fallen Bridge and Confluence. In this period, the UFSCar station had the maximum concentrations established by CONAMA Resolution 357/2005 for Cd (1.2 $\mu g.L^{-1}$), Cu^{++} (13.6 $\mu g.L^{-1}$) and Fe (1.78 $\mu g.L^{-1}$). There were also high levels of copper (19.2 $\mu g.L^{-1}$) and iron (0.58 $\mu g.L^{-1}$) at the Headwaters, Fallen Bridge and Confluence sites (Table 1). However, in April/04, the metal concentrations at all the sampling stations were within the limits established by CONAMA (the National Environmental Council). The origin of these metals can be directly associated with the industrial activities in the Monjolinho watershed. According to Campagna (2005), these activities, such as electroplating, mining, metallurgy and fabrication of plastic pipes use and generate residues of cadmium. Copper can be found in waste from hog and poultry breeding, from wood preservation and from water supply pipes. Besides this, cadmium, copper and other metals are utilized in the formulation of pesticides, which are widely applied to the local crops. The seasonal difference in the metal concentrations has a direct relation with the rainfall. In the dry period, the system receives a high load of effluents, which tend to accumulate because of the low water flow, unlike in the wet period, when these effluents tend to be diluted by the greater water volume. In the mainly agricultural areas (Fallen Bridge and Confluence), the high concentrations of some metals can be related to diffused pollution, associated with point sources along the urban stretch. Barreto (1999), in a previous study of the Monjolinho River, also detected the same metals and a decline in their concentrations in the wet period, corroborating our findings.

Regarding total nutrients (Table 2), the concentrations obtained were similar in July/03 and April/04, but different at the various sampling stations, with the lowest values at the Headwaters (18 $\mu g.L^{-1}$ in July/03 and 58 $\mu g.L^{-1}$ in April/04 for TP and 0.233 $mg.L^{-1}$ in July/03 and 0.286 $mg.L^{-1}$ in April/04 for NOT) and UFSCar (98 $\mu g.L^{-1}$ in July/03 and 108 $\mu g.L^{-1}$ in April/04 for PT and 0.606 $mg.L^{-1}$ in July/03 and 0.793 $mg.L^{-1}$ in April/04 for NOT). There were higher concentrations at Fallen Bridge (682 $\mu g.L^{-1}$ in July/03 and 644 $\mu g.L^{-1}$ in April/04 for TP and 3.5 $mg.L^{-1}$ in July/03 and 2.94 $mg.L^{-1}$ in April/04 for NOT) and Confluence (615 $\mu g.L^{-1}$ in July/03 and 465 $\mu g.L^{-1}$ in April/04 for TP and 4.01 $mg.L^{-1}$ in July/03 and 3.546 $mg.L^{-1}$ in April/04 for NOT), reflecting the influence of both the urban area and the contribution of the rural areas, from which the

Table 1. Heavy metals concentrations in water of Monjolinho River.

Heavy metals	Chrome ($\mu g.L^{-1}$)		Cadmiun ($\mu g.L^{-1}$)		Copper ($\mu g.L^{-1}$)	
	July/03	April/04	July/03	April/04	July/03	April/04
Headwaters	8.0	2.100	0.4	0	19.2	0
Ufscar	4.4	0.454	1.2	0.179	13.6	0
F. Bridge	8.4	9.097	0	0.417	21.2	4.916
Confluence	8.4	4.448	0	0.355	29.6	1.744
CONAMA 357/05	50.0		1.0		9.0	
	Zinc ($mg.L^{-1}$)		Iron ($mg.L^{-1}$)		Manganes ($mg.L^{-1}$)	
	July/03	April/04	July/03	April/04	July/03	April/04
Headwaters	0.027	0.0030	0.580	0.12	0.024	0.0086
Ufscar	0.170	0.0024	1.772	0.11	0.070	0.0072
F. Bridge	0.064	0.0048	5.089	0.34	0.200	0.0084
Confluence	0.063	0.0046	6.990	0.41	0.339	0.0222
CONAMA 357/05	0.18		0.3		0.1	

Table 2. Total phosphorus and organic nitrogen concentrations in water of Monjolinho River.

Sample stations	July/03		April/04	
	Total phosporus ($\mu g.L^{-1}$)	Total organic nitrogen ($mg.L^{-1}$)	Total phosporus ($\mu g.L^{-1}$)	Total organic nitrogen ($mg.L^{-1}$)
Headwaters	18	0.233	58	0.280
Ufscar	98	0.606	108	0.793
F. Bridgen	683	3.500	644	2.940
Confluence	615	4.010	465	3.546

fertilizers used on sugarcane crops may have been a source of nutrients in the river.

There was a tendency for the conductivity to increase from the Headwaters (area of least impact) in direction of the Confluence (inflow of urban, industrial and agricultural effluents) in July/03 (from 10 to 179 $\mu S.cm^{-1}$) and April/04 (from 8 to 116 $\mu S.cm^{-1}$), with lower values in the rainy period. In July/03 we recorded lower concentrations of dissolved oxygen than in the rainy period, related to the lower water flow, corroborating the conductivity data. Probably the low values of oxygen at Fallen Bridge (3.5 $mg.L^{-1}$ in July/03 and 4.2 $mg.L^{-1}$ in April/04) and the high concentrations of total nutrients and conductivity were the result of the excess decomposing organic material introduced into the system by raw sewage. Previous studies of the Monjolinho River have shown the same longitudinal variation for oxygen and conductivity at sampling stations located before and after the city of São Carlos (Mendes, 1998; Barreto, 1999; Marinelli, 2000).

The lowest water temperatures occurred in July/03 (18.6 °C at the Headwaters and 21.3 °C at UFSCar). In April/04, the lowest temperature was 20.3 °C at the Confluence and the highest was 23 °C at UFSCar. These readings can be related to the time of day when they were taken, since the surrounding areas of these sampling stations are characterized by pastures and sugarcane crops, with the exception of the Headwaters. The pH values did not differ between July/03 and April/04. The pH at the Headwaters (5.29 in July/03 and 5.65 in April/04) and at UFSCar (5.76 in July/03 and 5.93 in April/04) were slightly acidic. According to Salami (1996), this condition can, in general, be attributed to the decomposition of organic material from riverside vegetation, or due to the acidic characteristics of the soil in the region (Espíndola et al., 2000).

3.2. Physical and chemical analyses of the sediment

All the metals analyzed in this study were detected in the sediment of the Monjolinho River, except for Zn at the Headwaters in July/03. The highest values for the different metals, at all the sampling stations, were registered in July/03. Similar results were found for metals in the water. This fact may be associated with the low flows, which permits the metals to become associated with the particulate material, resulting in precipitation and accumulation in the river bed. According to Forstner (1990) and Burton (2001), this occurrence is related to the size of the particulate material present in the system. Ionic pollutants more easily adsorb to particulates with sizes up to 63 μm, such as silt and clay. Thus, sediment granulometric characteristics are important to interpret the behavior of pollutants. The highest concentrations were found for all the metals at the UFSCar sampling station, in July/03, with Cu^{++} (9.5 $mg.kg^{-1}$) and Cr (4.0 $mg.kg^{-1}$) standing out. These values can be associated with the granulometric composition of the sediment, which consists of roughly 20% silt and clay.

The sediments at the other sampling sites have a predominantly sandy composition. Therefore, at these places the metal concentrations were lower. In general, the highest concentrations were of Cu^{++} (minimum of 0.26 $mg.kg^{-1}$ at the Headwaters, in April/04, and maximum of 13.54 $mg.kg^{-1}$, in July/03, at the Confluence). According to the sediment guide for the protection of aquatic life prepared by the Canadian Council of Ministers of the Environment (CCME, 2003), the metal concentrations in the Monjolinho River were within the acceptable limits.

Considering the categories for classifying pollution of sediments by metals proposed by Thomas (1987), sediments of the Monjolinho River are classified as non-polluted, except for zinc (moderately polluted, in July/03 at UFSCar and Fallen Bridge), probably because of the lower adsorption of metals due to the greater presence of sand and lesser of silt and clay. Dornfeld et al. (2006), evaluating the same sampling stations, also associated the non-polluted sediment characteristic with the low concentrations of organic material and predominance of sand in the system. Campagna (2005), studying the characteristics of the sediment in the Monjolinho River and main tributaries, in different periods of the year, concluded that the river is subject to seasonal and spatial variations in relation to the level of pollution by metal compounds.

3.3. Toxicity tests with water samples in the laboratory

The results of the tests with *P. reticulata* adults and juveniles demonstrated toxicity at all the sampling sites in July/03, with similar responses, on a spatial scale, being for adults, since that mortality ranged between 45% (Headwaters) and 55% (Fallen Bridge). Juveniles were more sensitive, with mortality ranging from 55% (Headwaters) to 90% (UFSCar and Fallen Bridge) (Figure 3, Table 3). In April/04, despite the reduction in mortality for *P. reticulata* adults and juveniles, the samples were still toxic. Juveniles were always more sensitive than the adults, with mortality levels between 35% (Headwaters) and 50% (Fallen Bridge). Adult mortality ranged from 20% (Headwaters) to 40% (UFSCar) (Figure 3, Table 3). In July/03, the water at the UFSCar station was toxic to *D. rerio* juveniles (30% mortality). Although the differences in mortality between the adults and juveniles were small, the adults were more sensitive, showing toxicity at UFSCar and Fallen Bridge (both with 26.66% mortality). In April/04, we found no toxicity of the water samples for *D. rerio* juveniles and adults (Figure 3, Table 3).

The results of the ecotoxicological assays were compatibles with the limnological conditions of the river. The toxicity found in July/03 (Table 3, Figure 3), for the water samples from all sites for *P. reticulata* (juveniles and adults), demonstrated that the species is more sensitive to the pollutants in Monjolinho river than *Danio rerio* species. There was a toxicity response of the *D. rerio* adults at UFSCar, where the highest concentrations of metals in the water and sediment were found, and at Fallen Bridge, subject to strong influences of organic materials from the urban stretch of the river, with the presence of inorganic pollutants from rural areas. In April/04, histological analyses of the gills of test organisms exposed to the water samples showed some harmful effects, such as cell proliferation (in *P. reticulata* juveniles, at all the stations, and in *D. rerio* juveniles at UFSCar), fusion of secondary lamellae and thickening of the respiratory epithelium in the secondary lamellae (*P. reticulata* juveniles, at all sites). These are considered as level I alterations according Poleksic and Mitrovic-Tutundizc (1994) (Figure 5), which are reversible if the environmental conditions improve. Gills have a large surface area for exchange of gases between the blood and water (Newstead, 1987). Direct contact of the organ with the water facilitates the interaction with toxic substances, making the gills the primary organ where toxic effects occur (Hollis and Playle, 1997). Further, according to Perna-Martins (1997), in unfavorable conditions, there can be rearrangement of the gill epithelium, causing morphological and functional changes. A common alteration is increased thickness of the water-blood barrier, hampering the diffusion of oxygen (Perna-Martins, 1997). The cell membranes of the gills have a lipid bi-layer where there are potential binding sites for metals. The same is true for the proteins of membrane (Newman and Jagoe, 1994). Metals, once in contact with these binding sites, trigger various changes in tissues, such as fusion of lamellae, hyperplasia and hypertrophy. Therefore, although the fish did not die, considering the more sensitive phases of the organisms, histological and pathalogical analyses revealed indications of toxicity of the samples, which can be associated with the presence of metals, even at low concentrations, along with other toxic compounds.

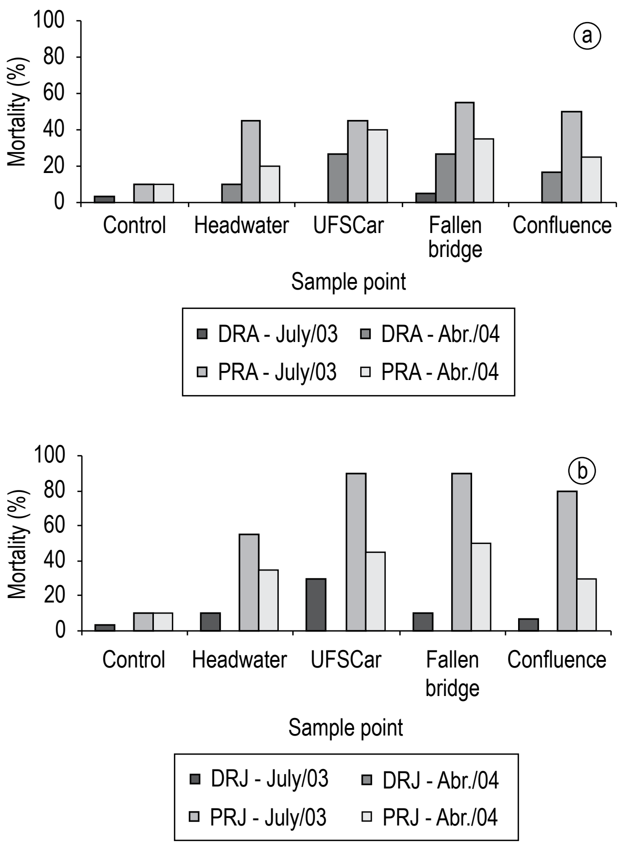

Figure 3. Comparative analysis of the mortality percentage for adult a) and juvenile b) fish, in toxicity tests with the water samples from the Monjolinho River (DRA: *D. rerio* adult; DRJ: *D. rerio* juvenile; PRA: *P. reticulata* adult and PRJ: *P. reticulata* juvenile).

3.4. In situ toxicity tests

According to Figure 4 and Table 4, the mortality level in July/03, for adults and juveniles of the two species, was 100% at Fallen Bridge and Confluence, demonstrating the toxicity at these sites. At the Headwaters, the juveniles

Table 3. P values after Fisher Exact Test and significant differences in mortality in the in situ tests in relation to the control group.

Sampling sites	P values (Fisher Exact Test)			
	D. rerio (adults)		*P. reticulata* (adults)	
	July/03	April/04	July/03	April/04
Headwater	1.5085	0.0033*	<0.0001*	0.0033*
UFSCar	NP	0.0004*	NP	0.0004*
Fallen Bridge	<0.0001*	NP	<0.0001*	NR
Confluence	<0.0001*	0.0033*	<0.0001*	0.0033*
	Danio rerio (juveniles)		*P. reticulata* (juveniles)	
	July/03	April/04	July/03	April/04
Headwater	<0.0001*	<0.0001*	<0.0001*	<0.0001*
UFSCar	NP	<0.0001*	NP	<0.0001*
Fallen Bridge	<0.0001*	NP	<0.0001*	NP
Confluence	<0.0001*	0.0033*	<0.0001*	0.0138*

* $p \leq 0.05$ –toxicity; NP- not performed.

of both species showed virtually the same behavior, with mortality of 66.66% for *D. rerio* and 70% for *P. reticulata*. Results for adults, were quite different, with only 3.33% mortality for *D. rerio* and 60% for *P. reticulata*. In April/04, the mortality levels were lower. The highest percentages were at UFSCar for both juveniles and adults of both species. Maximum mortality at this site was 80% for *P. reticulata* juveniles and 50% for *D. rerio* adults.

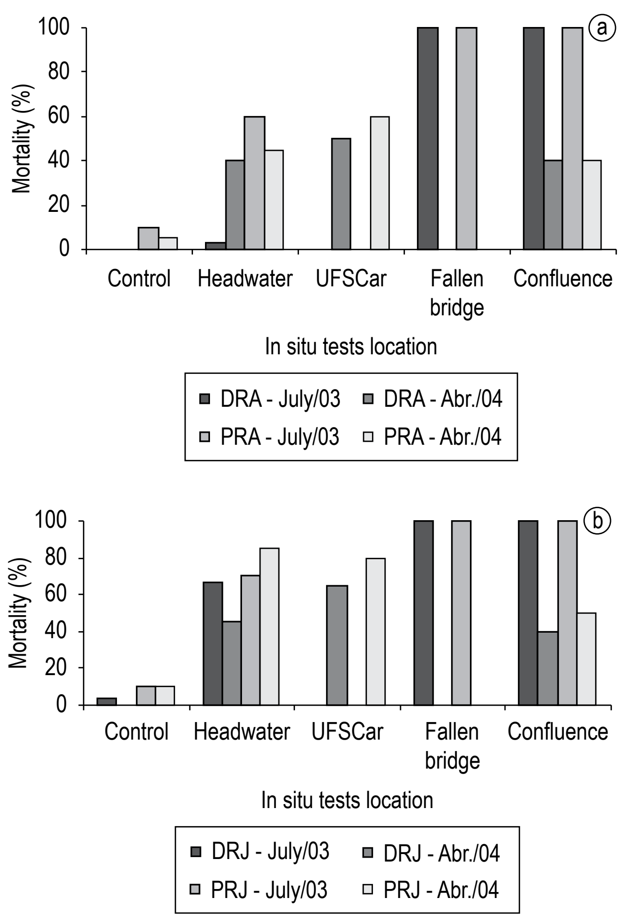

Figure 4. Comparative analysis of the mortality percentage for adults a) and juveniles b), in *in situ* toxicity tests. (DRA: *D. rerio* adult and DRJ: *D. rerio* juvenile; PRA: *P. reticulata* adult and PRJ: *P. reticulata* juvenile).

In this period, the *P. reticulata* juveniles were more sensitive, with greater mortality at the different stations, similar to the outcome of the laboratory tests. The responses obtained in the in situ tests demonstrated that the water at the different sites and in the two sampling periods can be characterized as toxic (Table 4), except at the Headwaters in July/03, where there was reduced mortality of *D. rerio* adults. Although the water quality of the Monjolinho River was better in April/04, this was not enough to protect the aquatic life, considering the toxic effects on the *P. reticulata* juveniles. These results show the importance of in situ tests, since they evaluate the dynamic processes in real time, with synergism or antagonism, that interfere in aquatic ecosystems, reflected in the greater or lesser toxicity to different organisms submitted to toxicity tests.

Vitozzi and de Angelis (1991), evaluating the sensitivity of different fish species exposed to 200 chemical compounds, found acute toxicity and species selectivity, i.e., each species has particular sensitivity to a determined substance. Many pollutants were not analyzed in the present study, but because of the farming activities in the region, they probably exist and can cause toxic effects in the system. Pélaez-Rodrigues (2001), for example, detected organochloride compounds in the water and sediments of the Monjolinho River at sampling stations near those in this study. Novelli (2005), Campagna (2005) and Fracácio (2006), examining the limnological characteristics of the Monjolinho River, also detected high concentrations of aldrin, heptachlor and endossulfan, including at the Headwaters. Organic compounds together with the metals and the limnological conditions found may have affected the metabolism and survival of the organisms in the in situ

Table 4. P values after Fisher Exact Test and significant differences in mortality in the water samples in relation to the control group.

Sampling sites	P values (Fisher Exact Test)			
	D. rerio (adults)		*P. reticulata* (adults)	
	July/03	April/04	July/03	April/04
Headwater	1000	1000	0.031*	0.6614
UFSCar	0.0046*	1000	0.031*	0.0648
Fallen Bridge	0.0046*	1000	0.0001*	0.1274
Confluence	0.052	1000	0.0004*	0.4075
	D. rerio (juveniles)		*P. reticulata* (juveniles)	
	July/03	April/04	July/03	April/04
Headwater	0.612	AM	0.0057*	0.1274
UFSCar	0.0122*	AM	0.0008*	0.031*
Fallen Bridge	0.612	AM	<0.0001*	0.138*
Confluence	1000	AM	<0.0001*	0.351

$^*p \leq 0.05$ – toxicity, AM: absence of mortality.

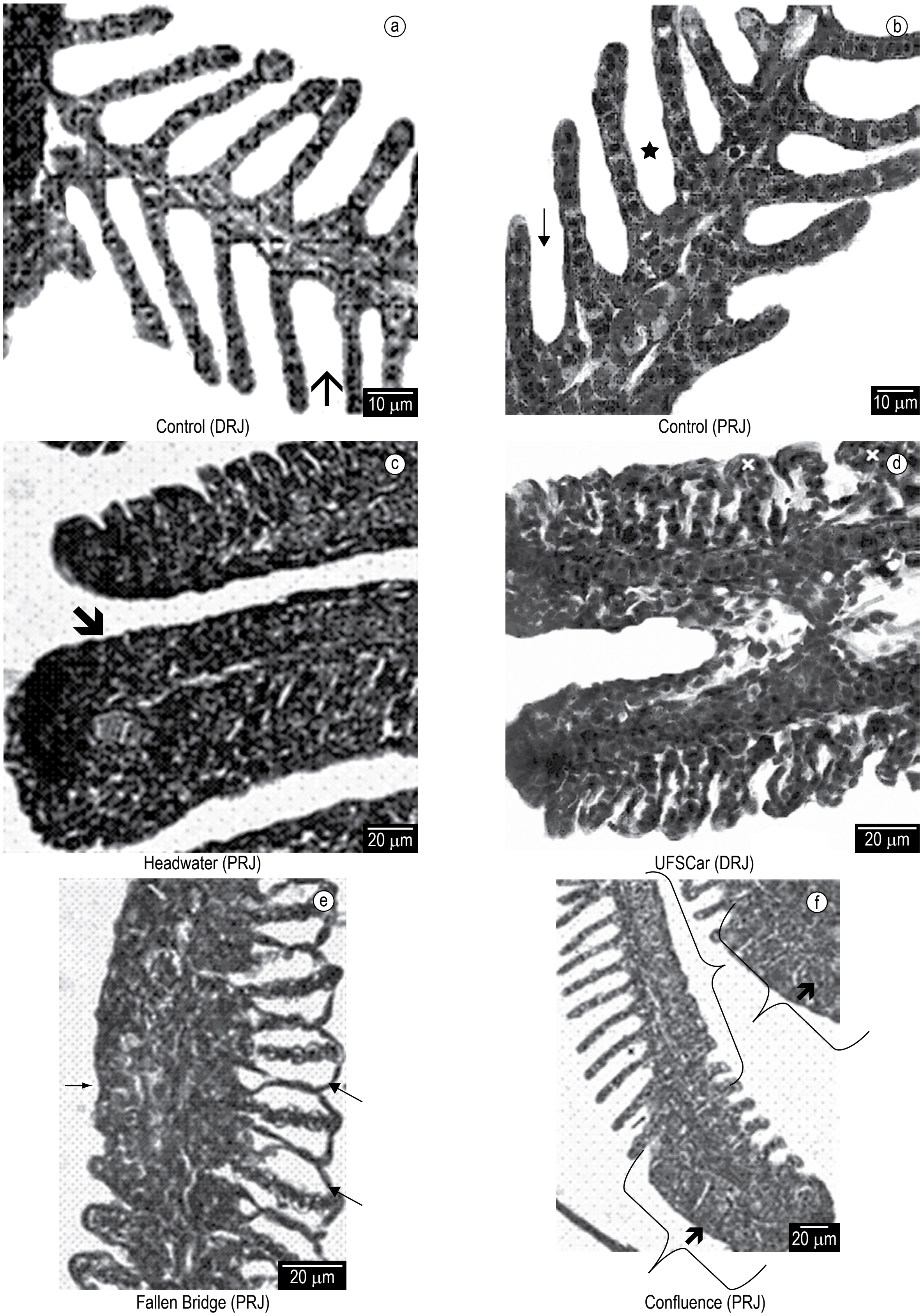

Figure 5. Histopathological changes in the surviving juvenile fish in laboratory toxicity tests with water samples. a, b) (Controls): well-defined epithelium (→) and space between secondary lamellae (➔); c): accentuated cell proliferation and fusion of secondary lamellae (arrow); d): hyperplasia (x) and reduced interlamellar spaces; e): detachment of the epithelial tissue (➔); f): accentuated cell proliferation (↗) with fusion of secondary lamellae (}).

tests. In July/03, at Fallen Bridge, the low concentrations of dissolved oxygen during the in situ test (3.59 $mg.L^{-1}$ at the start and 3.50 $mg.L^{-1}$ after 96 hours) may have caused the higher fish mortality.

Meletti (2003), studying the in situ toxicity with *Serrapinus notomelas* adults in the Piracicaba River Basin, found 100% mortality in a 24-hour period, attributing this fact to the low oxygen concentrations (maximum levels of 2 $mg.L^{-1}$) and to the toxic pollutants in the system. Furthermore, Meletti (2003) considered that the size of the holding chambers and the mesh opening could have contributed to the mortality levels. Here, for the tests with juveniles, the highest average weight per fish was obtained for *P. reticulata* in July/03 (0.093 g). Considering that the volume of water in the chambers was 900 mL, the experiment followed the recommendations of the Brazilian standards for tests with fish (1 g of fish per liter of water). For the tests with adults, the smallest average weight obtained per fish (0.1742 g, recorded for *D. rerio* in April/04) was above the limits of ABNT's protocol. However, limited space within the holding chambers did not seem to result in increased mortality in the present work. This may be evidenced by absence of significant mortality when the water conditions were better, such as in the Headwaters in July/03.

Another important aspect is the retention of particulate material by mesh opening, which could compromise water circulation within the chambers, causing oxygenation problems. But, retention did not result in increased mortality, because in both UFSCar and Confluence in April/04, where meshes were highly impregnated of particulate materials, mortality levels were lower than in Headwaters. However, we do not discard the necessity of repeating the experiments using chambers of varying volumes and mesh sizes, measuring the oxygen, pH and other variables in the water inside them in order to improve this poorly used, but clearly important method.

3.5. Comparison of the results

A comparison of the mortality results of the laboratory tests (7 days' exposure) with the in situ toxicity tests (96 hours) shows some similarities, such as lower mortality levels for the two species and phases (adults and juveniles) in April/04, associated with the better environmental conditions. This indicates that the two approaches are complementary. Another similarity was the greater sensitivity of *P. reticulata* than *D. rerio*. This finding was also found by Castro et al. (2004), in comparing *P. reticulata* and mosquitofish adults in in situ toxicity tests with acidic effluents. Measuring biochemical responses, *P. reticulata* was more sensitive to metals (Castro et al., 2004). In April/04, laboratory tests, in contrast to the in situ ones, indicated the absence of toxicity, except at UFSCar and Fallen Bridge, considering the mortality of *P. reticulata* juveniles. One hypothesis is that water samples are only a momentary reference of the river, since various intermittent conditions are not being evaluated. Tucher and Burton (1999) state that laboratory tests are not appropriate to evaluate the effects of non-point time-scale sources. In the present study, the responses to the in situ tests were more intense only at 96 hours of exposure, which showed the impacts on Monjolinho River. Dornfeld et al. (2006), evaluating the toxicity of the Monjolinho River through in situ and laboratory tests with *Chironomus xanthus* (96 hours), at the same sampling stations, also recorded higher mortality in the *in situ* tests. They associated this data to the low oxygen concentrations and high pollutant loads in the river.

3.6. Histological analyses

Besides mortality, histological biomarkers have also been used to help interpret toxicity tests and to understand the conditions of complex systems (Castro et al., 2004). According to Laurant and Perry (1991), species undergo morphological changes to adapt to new environmental conditions. Therefore, we carried out histological analyses in April/04 to evaluate whether, even with the improvement in the water quality and the decline in mortality, the surviving fish were suffering any harmful effects. Results show that even with the short exposure time, *P. reticulata* juveniles and adults underwent tissue changes in comparison with the control organisms. This was not the case for *D. rerio* adults and juveniles, indicating the greater sensitivity of *P. reticulata* to the conditions of the Monjolinho River (Figure 6). The only alteration noted in *D. rerio* was hyperplasia of the secondary lamellae of adults. *P. reticulata* adults were more resistant than juveniles regarding mortality, although in the conducted tests, samples at the Confluence caused gill alterations, such as aneurisms, which compromised respiration. The *P. reticulata* juveniles also showed accentuated cell proliferation, fusion of lamellae and thickening of the respiratory epithelium (Figure 6). According to Sorensen (1991), the exposure of *Funduculus hetericlitus* to 50 ppm of cadmium for 48 hours caused gill alteration such as hypertrophy. Bilinks and Jonas (1973) reported that the exposure of *Salmo gairdneri* to 64 ppb of Cu^{++} for 48 hours caused fusion of secondary lamellae. Karlsson-Norrgren et al. (1985) found that after exposure for six weeks to 10 $\mu g.L^{-1}$ of Cd, *D. rerio* adults suffered enlarged secondary lamellae, with the interlamellar spaces filled in, resulting in reduced capacity for gas diffusion. Some metals, such as Zn, Fe, Mn, Cd, Cu^{++}, Al, Ni, Co, Ag, Au and Hg, induce the production of mucous, an attempt by the organism to protect the gill respiratory epithelium, which can cause asphyxia (Sorensen, 1991). In the present work, the presence of metals may have contributed to the gill tissue changes and mortality of the fish. There were similarities in the histological changes of the fish subjected to the laboratory and in situ tests, except in *P. reticulata* juveniles, which presented different changes in the laboratory and in situ (Figures 5 and 6).

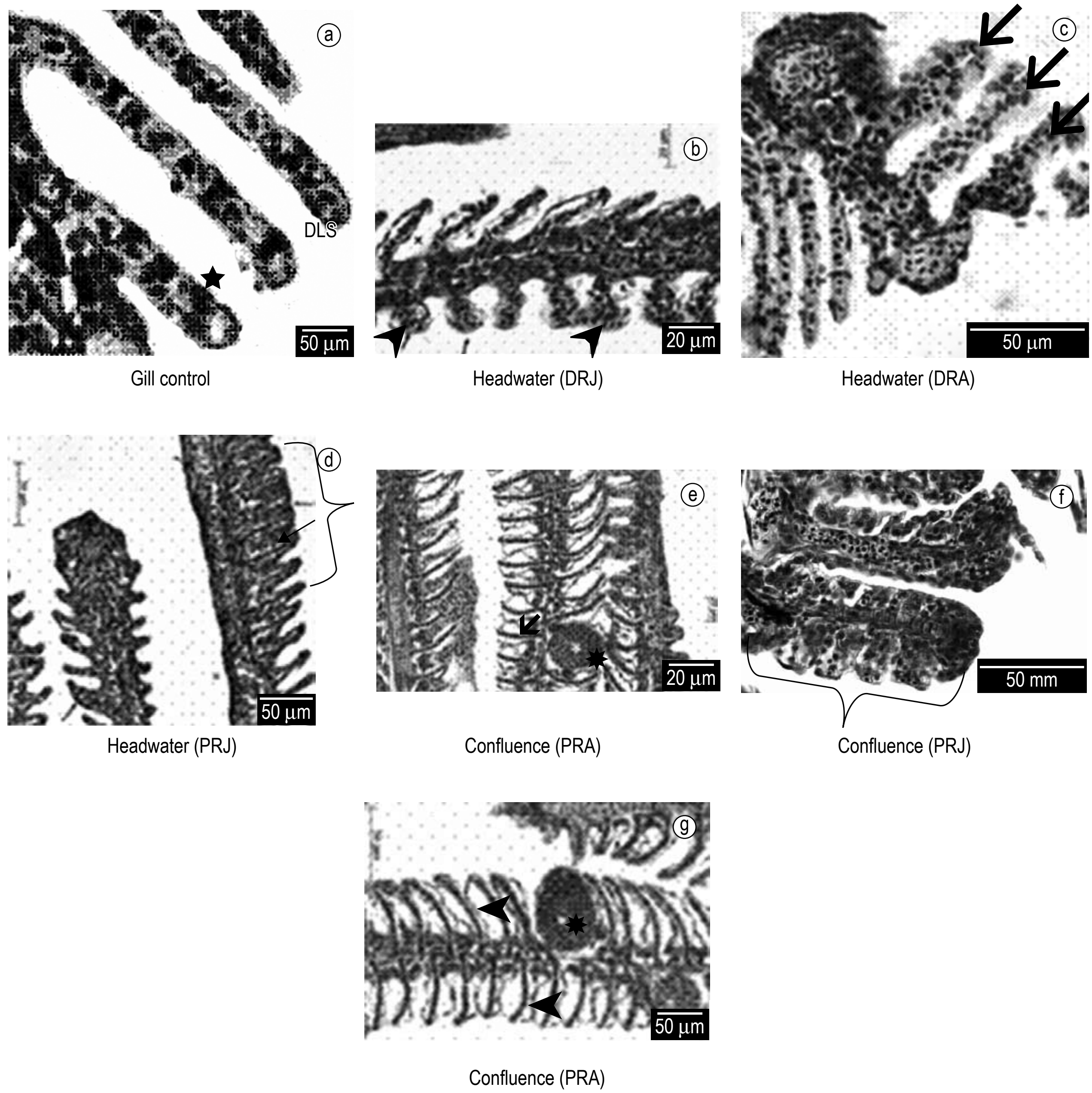

Figure 6. Histopathological changes in the surviving juvenile fish in *in situ* toxicity tests. A (Control): good development of the secondary lamellae (DSL), well-defined space between them and juxtaposed epithelium (★), B- cell proliferation at the tips of the secondary lamellae, (➤); C: hyperplasia in the secondary lamellae (➔), D: cell proliferation in the primary and secondary lamellae (→), with fusion of the secondary ones (}), E: detachment of the epithelial tissue (→) and aneurisms (✸), F: cell proliferation with fusion of the secondary lamellae (}) and G: thickening of the respiratory epithelium in the secondary lamellae (❮)and aneurisms (✸).

4. Conclusions

Monjolinho River showed physical and chemical changes due to seasonal variations (dry and wet periods), which were reflected in data of laboratory and in situ tests with *D. rerio* and *P. reticulata* adults and juveniles. *P. reticulata* was more sensitive than *D. rerio*. Results of in situ tests indicated the toxicity of the water of the Monjolinho River, in both periods, which did not occur in the laboratory tests in April/04, indicating the importance of in situ tests to evaluate water quality. Histological analyses were complementary tools to help confirm and interpret the environmental conditions in the river.

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References

Associação Brasileira de Normas Técnicas – ABNT. *NBR 7181*. Análise granulométrica de solos. Rio de Janeiro, 1984. 13p.

Associação Brasileira de Normas Técnicas – ABNT. *NRB 12714*. Água: ensaio de toxicidade aguda com peixes. Parte I - Sistema Estático. Rio de Janeiro, 1993. 15p.

Associação Brasileira de Normas Técnicas. *Projeto 00:001.44-001*. Ecotoxicologia aquática: toxicidade aguda – Método de ensaio com peixes. Rio de Janeiro, 2003. 18p.

BARRETO, AS. *Estudo da distribuição de metais em ambiente lótico, com ênfase na assimilação da das comunidades biológicas e a sua quantificação no sedimento e água.* São Carlos: Universidade de São Paulo - USP, 1999. Tese de Doutorado.

BURTON Jr., GA. Assessing the toxicity of freshwater sediments. *Environm. Toxicol. Chem.* 1991, vol. 10, no. 3, p. 1585-1627.

BURTON Jr., GA. *Sediment toxicity assessment*. 1 ed. Chelsea: Lewis Publishers, 1992. 158p.

Canadian Council of Ministers of the Environment - CCME. *Summary of Existing Canadian Environmental Quality Guidelines*: Summary Table. New York, 2003. Available from: <http://www.ccme.ca/assets/pdf/e1_062.pdf>. Access in: 12 de Novembro de 2004.

CAMPAGNA, AF. *Toxicidade dos sedimentos da bacia Hidrográfica do rio monjolinho (São Carlos – SP)*: ênfase nas substâncias cobre, aldrin e heptachloro. São Carlos: Universidade de São Paulo – USP, 2005. Dissertação de Mestrado.

CASTRO, BB., SOBRAL, O., GUILHERMINO, L. and RIBEIRO, AR. In situ bioassays of individual and biochemical responses using small fish species. *Ecotoxicol.* 2004, vol. 13, no. 4, p. 667-681.

Companhia de Tecnologia de Saneamento Ambiental – CETESB. *Projeto 11.01.00*. Desenvolvimento de metodologia para avaliação da qualidade ambiental. Metodologia para avaliação da toxicidade crônica com o peixe Brachydanio rerio. São Paulo, 1994. 19p.

CHAPPIE, DJ. and BURTON, GAJR. Applications of aquatic and sediment toxicity testing in situ. *Journ. Of Soil Contamin.* 2000, vol. 9, no. 4, p. 322-341.

Conselho Nacional do Meio Ambiente – CONAMA. *Resolução 357* de 17 de março de 2005. Available from: <www.mma.sp.gov.br/conama/35705>.

DORNFELD, CB., ESPÍNDOLA, ELG., FRACÁCIO, R., RODRIGUES, BK. and NOVELLI, A. Comparação de bioensaios laboratoriais e "in situ" utilizando *Chironomus xanthus* na avaliação da toxicidade de sedimentos do rio Monjolinho (São Carlos, SP). *J. Braz. Soc. Ecotoxicol.* 2006, vol. 2, no. 1, p. 161-165.

ESPÍNDOLA, ELG. O Rio Monjolinho: um estudo de caso. In ESPÍNDOLA, ELG., SILVA, JSV., MARINELLI, CE. and ABDON, MM. (Eds.). *A bacia hidrográfica do Rio do Monjolinho*. São Carlos: Rima Editora, 2000. p. 36-40.

FORSTNER, U. Inorganic sediement chemistry and elemental speciation. In BAUDO, R., GIESY, JP. and MUNTAU, H. (Eds.). *Sediments*: chemistry and toxicity of in-place pollutants. London: Lewis Publishers, Inc., 1990. p. 61-97.

FRACÁCIO, R., RODGHER, S., ESPÍNDOLA, ELG., PASCHOAL, CMRB., LIMA, D., NASCIMENTO, AP. and RODRIGUES, MH. Abordagem ecotoxicológica. In ESPÍNDOLA, ELG., SILVA, JSV., MARINELLI, CE. and ABDON, MM. (Eds.). *A bacia hidrográfica do Rio do Monjolinho*. São Carlos: Rima Editora, 2000. p. 104-113.

GUERESCHI, RM. *Monitoramento biológico da bacia hidrográfica do rio Monjolinho pelo uso de invertebrados bentônicos*. São Carlos: Universidade Federal de São Carlos - UFSCar, 1995. 105p. Monografia em Biologia.

HOFFMAN, DJ. Wildlife toxicity tests. In HOFFMAN, DJ., RATTNER, BA., BURTON Jr., GA. and CAIRNS Jr., J. *Handbook of Ecotoxicology*. 2 ed. New York: Lewis Publishing, 2002. p. 47-69.

HOLLIS, L. and PLAYER, RC. Influence of dissolved organic matter on copper binding, and calcium on cadmium binding, by gills of Rainbow trout. *J. Fish. Biol.* 1997, vol. 50, no. 6, p. 703-720.

KARLSSON-NORRGREN, L. Cadmium-induced changes in gill morphology of the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan), and Rainbow trout, *Salmo gairdineri*. *J. Fish Biol.* 1985, vol. 27 no 1, p. 81-95.

LLOYD, R. *Pollution and freshwater fish*. Oxford: Fishing News Books, 1992. 183p.

MARINELLI, CE., MORETTO, EM., BRUCHA, G. and LUCCA, JV. Limnologia. In ESPÍNDOLA, ELG., SILVA, JSV., MARINELLI, CE. and ABDON, MM. (Eds.). *A bacia hidrográfica do Rio do Monjolinho.* São Carlos: Rima Editora, 2000. p. 90-111.

MATSUI, S. *Diretrizes para o gerenciamento de lagos*: gerenciamento de substâncias tóxicas em lagos e reservatórios. São Carlos: Rima Editora, 2002. 245p.

MELETTI, PC. and ROCHA, O. Development of a chamber for in situ toxicity tests with small fishes. *Braz. J. Biol.* 2002, vol. 62, no. 1, p. 187-190.

MELETTI, PC. *Avaliação da degradação ambiental por meio de testes de toxicidade com sedimento e de análises histopatológicas em peixes*. São Carlos: Universidade de São Paulo - USP, 2003. Tese de Doutorado.

MENDES, AJS. *Avaliação dos impactos sobre a comunidade macrozoobentônica, no córrego do Monjolinho (São Carlos, SP).* São Carlos: Universidade de São Paulo - USP, 1993. Dissertação de Mestrado.

MEREGALLI, G., VERMEULEN, AC. and OLLEVIER, F. The use of Chironomidae deformation in an in situ test for sediment toxicity. *Ecotox. and Environ. Saf.* 2000, vol. 47, p. 231-238.

NEWMAN, MC. and JAGOE, CH. Ligands and bioavailibility of metals in aquatic environments. In HAMELINK, JL., LANDRUN, PF., BERGMAN, HL. and BENSON, WH. (Eds.). *Bioavailability, physical, chemical, and biological interactions*. London: Lewis Publishers, 1994. p. 39-62.

NEWSTEAD, JD. Fine structure the respiratory lamellae of Teleostean gills. *Zeitsehrif fur Zelfforshung*, 1987, vol. 70, no. 3, p. 420-445.

NOVELLI, A. *Estudo limnológico e ecotoxicológico da água e sedimento do rio Monjolinho – São Carlos (SP), com ênfase nas substâncias de referência cádmio e cobre*. São Carlos: Universidade de São Paulo - USP, 2005. 212p. Dissertação de Mestrado.

PELAÉZ-RODRIGUES, M. *Avaliação da qualidade da água da bacia do Alto Jacaré-Guaçu / SP (Ribeirão do Feijão e Rio do Monjolinho) através de variáveis físicas, químicas e biológicas.* São Carlos: Universidade de São Paulo - USP,2001. 328p. Tese de Doutorado.

POLEKSIC, V. and MITROVIC-TUTUNDIZIC, V. Fish gills monitor of sublethal and chronic effects of pollution. In MULLER, R. and LLOYD, R. (Eds.). *Sublethal and Chronic effects of Pollutants on freshwater fish.* London: Fishing News Books, 1994. p. 339-352.

PERNA-MARTINS, SA. *Efeito das concentrações de* Na^{+}, Cl^{-}, *e* Ca^{+2} *sobre a morfologia e ultra – estrutura das brânquias de cascudo, Hypostomus plecostomus (Teleostei, Laricariidae).* São Carlos: Universidade Federal de São Carlos - UFSCar, 1997. 243p. Tese de Doutorado.

REBOUÇAS, AC. Água Doce no Mundo e no Brasil. In REBOUÇAS, AC., BRAGA, B. and TUNDISI, JG. (Eds.). *Águas Doces no Brasil*: capital ecológico, uso e conservação. São Paulo: Editora Escrituras, 1999. p. 1-64.

RINGWOOD, AH. and KEPPLER, CJ. Comparative in situ and laboratory sediment bioassays with juvenile *Mercenaria mercenaria*. *Environm. Toxicol.* 2002, vol. 21, no. 8, p. 1651–1657.

ROSA, F. and AZCUE, JM. *Peeper methodology*: a detailed procedure from field experience. Burligton: NWRI, 1997. 12p.

SALAMI, LNBP. *Estudo das influencias climáticas e antropogênicas nas características fisico-químicas no rio Monjolinho.* São Carlos: Universidade de São Paulo - USP, 1996. 169p. Dissertação de Mestrado.

São Paulo State Decree 10.755 of November 22, 1977. *Provides the classification of water bodies according to Decree 8468 of September 8, 1976.* Brasil, 1977. 25p.

Secretaria Especial do Meio Ambiente - SEMA. Avaliação da toxicidade para peixes (D.3). In *Manual de testes para avaliação da ecotoxicidade de agentes químicos*. Brasília, 1988. 10p.

SÉ, JA. *O rio Monjolinho e sua bacia hidrográfica como integradores de sistemas ecológicos*: um conjunto de informações para o início de um processo de pesquisas ecológicas, de educação e gerenciamento ambientais a longo prazo. São Carlos: Universidade de São Paulo - USP, 1992. Dissertação de Mestrado.

SORENSEN, EMB. *Metal poisoning in fish*. New York: Boca Raton Press, 1991. 322p.

THOMAS, RLA. Protocol for selection of process-oriented remedial options to control *in situ* sediment contaminants: ecological effects of *in situ* sediment contaminants. *Hydrobiologia*, 1987, vol. 49, no. 3, p. 247-258.

TORRE, FRLA., FERRARI, L. and SALIBIÁN, A. Long-term in situ bioassays of the Reconquista River (Argentina) water with *Cyprinus carpio* as sentinel organism. *Wat. Air and Soil Pollut*. 2000, vol. 121, no. 5, p. 205-215.

TUCHER, KA. and BURTON Jr., GA. Assessment of nonpoint-source runoff in a stream using in situ and laboratory approaches. *Environm. Toxicol. Chem.* 1999, vol. 18, no. 12, p. 2797-2803.

VITOZZI, L. and De ANGELIS, G. A critical review of comparative acute toxicity data on freshwater fish. *Aquatic. Toxicol.* 1991, vol. 19, no. 2, p. 167-204.

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