Research Article

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Biomarkers for *Ex Situ* Ni and Pb Exposure in Common Carp (*Cyprinus carpio* L.)

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Abstract: This work aimed to study the effects of heavy metal exposure on the respiration rate and histological structure of common carp (*Cyprinus carpio* L.) gills. Fish were treated with different soluble concentrations of Ni and Pb in laboratory conditions for a total acute period of 72 hours. The metal concentrations were prepared as 75, 50 and 25% of the maximum permissible levels (100%) set by law. The results showed a higher index of respiration rate in the fish from all experimental tanks for both metals, compared to the control in the beginning of the experiment, but there was no pattern of increase or decrease in relation to the metal concentrations. After 72 hours of exposure we observed the same pattern, but in addition the respiration rate of the fish in the tanks treated with Pb showed an increase in a dose-dependent manner. We also observed different histological changes in the gill epithelium, which included proliferative and degenerative changes, as well as changes in the circulatory system. In addition, the degenerative changes were more pronounced in the fish, treated with Pb concentrations, and the blood circulatory system showed mainly vasodilatation, which caused pathological changes in the gills. In sum, we can conclude that Ni and Pb have severe effects on the respiration rate and gill histology of common carp, even at concentrations, which were lower than the allowable ones.

Key words: heavy metals, fish, respiration rate, histology, gills

Introduction

The aquatic systems may extensively be contaminated with heavy metals released form domestic, industrial and other anthropogenic activities (Conacher *et al*. 1993, Vinodhini, Narayanan 2008). Heavy metal contamination have negative effects on the ecological balance and diversity of aquatic organisms (Vosyliene, Jankaite 2006), and may pose serious threat to the survival of aquatic fauna, including fish (Mishra, Mohanty 2009, Poleksic *et al*. 2010).

Measurements of different biomarker responses in fish provide information that can contribute to environmental monitoring programs designed for various aspects of environmental risk assessment (Van der Oost *et al*. 2003). According to Chen, Yang (2007), Dobreva *et al*. (2008), Nishida (2011) the toxic effects of heavy metals may be linked to changes in the respiration rate and may also be used as an indicator of polluted waters. Changes in the respiration rate are associated with insufficient amount of oxygen in the water, causing stress to the fish. Fish gills are the main target of many aquatic pollutants, thus being an excellent model to examine the effects of dissolved substances and are also the most seriously affected organ due to their constant contact with the water (Biagini *et al.* 2009). Moreover, histopathological biomarkers incorporate biotic factors and water quality, so they are reliable markers of environmental stress (Stentiford *et al.* 2003, Zimmerli *et al.* 2007, Padmini, Usha Rani 2010).

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Common carp (*Cyprinus carpio*, L.) is an economically important fish species and probably in recent years, it has been the most used freshwater species in aquaculture all over the world. It has also been proposed as a bioindicator in many toxicological assays because it is relatively insensitive and can survive and bioaccumulate contaminants even at heavily polluted sites (Ye *et al*. 2008, Ondarza *et al*. 2010).

In the present experiment the main purpose was to study the possible effects of decreasing concentrations of Ni and Pb in laboratory conditions for a total acute period of 72 hours on common carp (*Cyprinus carpio* L.). These metals are considered as priority substances in surface waters according to Directive 2008/105/EO of the European parliament and the Council. For this aim we chose two biomarkers: 1) to measure the respiration rate and 2) to investigate the gills histological structure, as well as to present the degree of expression of each histological lesion.

Material and Methods

Experimental setup

Fish were obtained from the Institute of Fisheries and Aquaculture, located in the city of Plovdiv, Bulgaria. They were of the same size-group (21.02 gr ± 13.15; 11.73 cm ± 2.16) with no external pathological abnormalities. After transportation the fish were moved in glass aquaria with 50 L chlorine-free tap water (by evaporation) to acclimatize for four days. The individuals were divided into nine test groups (n=10), including control. They were treated with different $Ni(NO_3)_2$ and $Pb(NO_3)_2$ concentrations, which were prepared as 75, 50 and 25% of the maximum permissible levels (100%) set by the national law. Fish were not fed prior or during the experiment. The physico-chemical characteristics of the aquarium water such as: pH, temperature, oxygen level and conductivity were measured once on the 0, 24th and 72nd hour according to a standard procedure with a combined field-meter (APHA 2005). All experiments were conducted in accordance with national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes according to Directive 2010/63/EU.

Respiration rate

The respiration rate was measured at the beginning of the experiment (0-hour) and at the end of the experiment (72nd hour). At the given time 5 fish were transferred in 30 L glass tanks filled with water from the test aquariums. The oxygen levels were measured, using oximeter "Oxi 315i/SET". The tanks were filled with water to the edge of the tanks and covered with plastic foil in order any oxygen transfer from the air to be eliminated. The tanks were left for one hour and thereafter, the oxygen level was measured once again. The respiration rate was calculated by determining the difference in the dissolved oxygen levels before and after the passed time, following Tsekov (1989):

$$I = \frac{Q_2}{G},$$

where I – respiration rate index; G – weight of the fish, in grams, Q_2 – oxygen consumed by the mussels between the two measurements (the difference between the oxygen levels before and after the 1-hour Q_2=Q-Q_1 hour).

Q was calculated by the following formula:

$$Q = V \times q,$$

where: Q – total oxygen level in the tank; V – volume of the water in the tank, in liters; q – level of dissolved oxygen in 1 liter of water (mg/L).

For the statistical processing of the data the software package "Statistica 7.0" (StatSoft Inc. 2004) was used. T-test was applied to study the difference between the respiration rate of the tested fish and control, respectively. Significance level was set at 0.05.

Histopathological analysis

Fish dissection was performed according to the international standard procedures given in the EMERGE Protocol (Rosseland *et al*. 2003). All histological were placed prepared according to a standard procedure for light light microscopy analysis (Romeis 1989).

Semi–quantitative scoring

The histological alterations in the gill epithelium were determined semi–quantitatively by using the grading system of Pierce *et al*. (1978) and Zimmerli *et al*. (2007), which we combined and modified. Each grade represented specific histological characteristics and was categorized as follows: (0) – no histological alterations, which represented normal histological structure; (1) – mild histological alterations; (2) – moderate histological alterations; (3) – severe histological alterations; (4) – very severe histological alterations in the gill surface architecture.

Results and Discussion

Respiration rate

The results for the physico-chemical properties of the water from all experimental tanks, including the control are presented in Table 1. The pysico-chem-

ical properties were similar between the 0, 48th and 72nd hour. These for the control group were as follows: pH – 8.1; conductivity – 435 μS/cm, oxygen level 6.5 mg/L and temperature – 21.5 °C, respectively. Therefore, we believe that the changes, which we found in the respiration rate and gill histological structure, are not due to changes in the pH, oxygen level, temperature or conductivity.

The results for the respiration rate are presented in Table 2. At the beginning of the experiment (0 hour) there was no visible pattern in the changes in the respiratory rate index for both metals, although the fish from all concentrations showed a higher respiratory rate index, compared to the control. At the end of the experiment (72 hours of exposure) there was a visible pattern of increase of the respiration rate index with the increasing concentrations of both metals and again all test values were much higher than the control.

In most cases the respiration rate increases with the increase of the pollutant concentration and level of toxicity (Kumar *et al*. 2012).

The reason for this is that the organism tries to deliver more oxygen to all tissues and organs triggered by the stress, which is caused by the toxic exposure. This was also the case with the fish in the present experiment they reacted by increasing the respiration rate with the increasing metal concentrations after the 72nd hour of exposure. On the other hand, Dobreva *et al*. (2008) studied how increasing concentrations of $CuSO_4 5H_2O$ affected the respiration rate of *Carassius gibelio* and observed 30-45 % decrease in the respiration rate corresponding to the experimental concentrations. Ivanova *et al*. (2008) also tested how sewage water influenced the respiration rate of prussian carp (*Carassius auratus gibelio*) and common carp (*Cyprinus carpio*), and found that the respiration rate of both fish species decreased compared to the control.

Histopathological analysis

No histopathological changes were observed in the control fish gills. Gill histological structure of the control carps is shown in Figure 1A. With regards to the proposed grading system, the control common carp gill histological characteristics were evaluated as relatively normal (-).

On the other hand, the histopathological results indicated that the different metal concentrations caused morphological alterations in the gill epithelium and the blood circulatory system. We found lamellar proliferative changes such as lamellar lifting, edema, proliferation of the filamentous epithelium, glandular cells, epithelial cells of the secondary lamellae and cartilage tissue, fusion and degenerative changes in the gill epithelium. In addition, we found vasodilatation in the blood circulatory system (Table 3, Fig. 1). Histological alterations in fish gills are also presented in the work of Figueiredo-Fernandes *et al*. (2007), Van Dyk *et al*. (2007), Vinodhini, Narayanan (2009), and Jabeen, Chaudhry (2010), who suggested that they can be used as biomarkers for heavy metal contamination

In addition, the degree of expression of lamellar lifting and edema caused by the increasing concentrations of Ni and Pb was severe and very severe (Fig. 1E).

Similarly to us, Schwaiger *et al.* (1997) Pane *et al*. (2004) and Figueiredo-Fernandes *et al*. (2007),

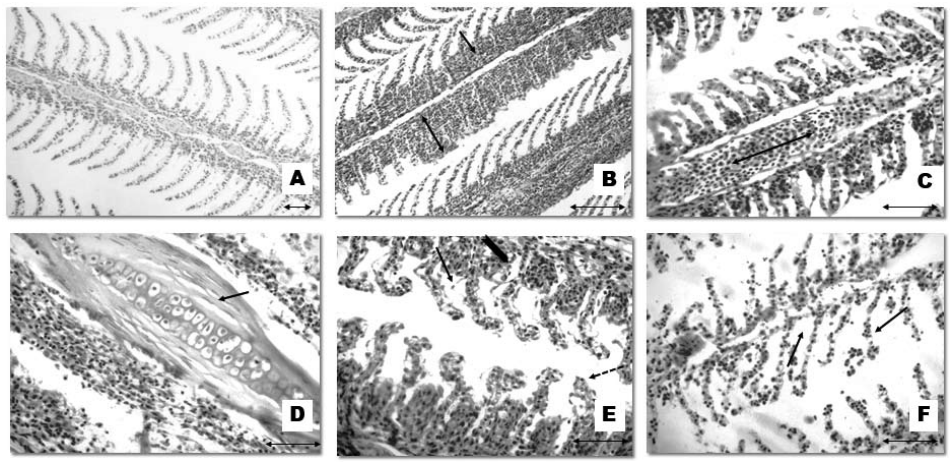

Fig. 1. Histopathological alterations in *Cyprinus carpio* L. exposed to different Ni and Pb concentrations: A-control group, x200; B-Proliferation of filamentous epithelium (⟶) and fusion (⟷) (500 μg Ni), x400; C-vasodilatation of venous sinus (750 μg Ni), x400; D-cartilage tissue proliferation (750 μg Ni), x400; E-lamellar lifting (⟶), edema (⇢) and proliferation of cartilage tissue (➤) (90 μg Pb), x400; F-degeneration of gill epithelium (360 μg Pb), x400

Table 1. Water physico-chemical properties in the experimental tanks, results for the 0, 24th and 72nd hour (average ± SD)

Metal concentrations, µg for 50 L tanks	pH	Conductivity, µS/cm	Temperature, ˚C	Oxygen level, mg/L
Ni 1000	7.98 ± 0.5	492 ± 1.5	21.75 ± 1	6.3 ± 0.5
Ni 750	8.1 ± 0.5	490 ± 1	20.75 ± 1.5	6.3 ± 0.3
Ni 500	8.15 ± 0.5	489.5 ± 0.5	22.75 ± 1.3	6.5 ± 0.5
Ni 250	8.02 ± 0.3	489 ± 0.5	22 ± 0.5	6.6 ± 0.1
Pb 360	8.12 ± 0.5	486.5 ± 0.5	23.5 ± 0.5	6.2 ± 0.5
Pb 270	8.13 ± 0.3	488 ± 0.3	22.75 ± 0.5	6.4 ± 0.5
Pb 180	8.2± 0.5	483.5 ± 0.3	21.75 ± 0.5	6.5 ± 0.3
Pb 90	8.195 ± 0.3	480.5 ± 1.5	21.75 ± 0.3	6.5 ± 0.3

Table 2. Index of respiration rate of common carp (*Cyprinus carpio*) exposed to different Ni and Pb concentrations at the beginning of the experiment (0 hour) and the end of the experiment (72nd hour).

Metal concentrations, µg for 50 L tanks	Water volume, L	Weight,g (G)	Total oxygen level (mg/L)					Index of respiration rate (I)
			Beginning		End		Total	
			q	Q	q_{1h}	Q_{1h}	(Q_2)	
Beginning (0 hour)								
Control	30	102.95	7.9	237.0	7.7	231.0	6.00	0.058
Pb 90	30	123.89	6.8	204.0	5.1	153.0	51.00	0.412
Pb 180	30	108.83	6.6	198.0	5.2	156.0	42.00	0.386
Pb 270	30	92.14	6.5	195.0	5.3	159.0	36.00	0.391
Pb 360	30	106.90	6.5	195.0	5.6	168.0	27.00	0.253
Ni 250	30	146.20	7.8	234.0	7.3	219.0	15.00	0.103
Ni 500	30	114.77	7.8	234.0	7.1	213.0	21.00	0.183
Ni 750	30	105.37	7.5	225.0	6.9	207.0	18.0	0.171
Ni 1000	30	115.16	7.2	216.0	6.7	201.0	15.0	0.13
72nd hour								
Control	30	128.78	6.0	180.0	5.5	165.0	15.00	0.116
Pb-90	30	169.49	7.5	225.0	4.7	141.0	84.00	0.496
Pb-180	30	119.61	7.5	225.0	4.9	147.0	78.00	0.652
Pb-270	30	106.81	7.6	228.0	5.3	159.0	69.00	0.646
Pb-360	30	112.91	7.7	231.0	5.2	156.0	75.00	0.664
Ni-250	30	140.09	7.6	228.0	7.2	216.0	12.00	0.086
Ni-500	30	98.39	7.3	219.0	6.8	204.0	15.00	0.152
Ni-750	30	102.38	7.4	222.0	6.7	201.0	21.00	0.205
Ni-1000	30	104.15	7.4	222.0	6.5	195.0	27.00	0.259

consider that the most frequently detected changes in the gills of carp under the heavy metal exposure are lamellar lifting and edema. They can serve as a protection mechanism as the secondary lamellae epithelium is lifted from the gills, which increases the distance over which contaminants have to pass to reach the bloodstream (Arellano *et al.* 1999). In addition, like Wangsongsak (2003) and Hassan (2011) we think believe edema is possibly due to increased capillary permeability.

Proliferation of the filamentous epithelium, secondary lamellae and glandular cells showed differences in the degree of expression between the two heavy metals (Table 3, Fig. 1B, D). The results suggest that Pb caused more pronounced proliferative changes compared to Ni. Like Thophon et al. (2003) and Georgieva et al. (2014) we consider that the proliferative changes in the gills could be protective mechanisms which lead to an increase in the distance between the external environment and the blood, and thus serve as a barrier to penetration of toxicants. We can summarize that the degree of proliferative changes more severe in the fish treated with Ni.

At the same time, a higher degree of fusion was observed in the fish treated with Ni (Table 3). This suggests that Ni activated more intensive prolifera-

Table 3. Histopathological alterations in common carp (*Cyprinus carpio)* gills exposed to different Ni and Pb concentrations after 72 hours

Histopathological alterations in *C. carpio* gills	**Control group**	**Ni (µg)**				**Pb (µg)**			
		250	**500**	**750**	**1000**	**90**	**180**	**270**	**360**
Lamellar lifting	1	3	3	4	4	3	3	3	4
Edema	0	3	3	2	3	1	2	3	4
Proliferation of filamentous epithelium	0	3	3	3	3	2	3	4	3
Proliferation of glandular cells	0	1	0	0	0	0	1	2	1
Proliferation of epithelial cells of secondary lamellae	0	2	0	0	0	2	3	2	2
Proliferation of cartilage tissue	0	0	2	1	2	2	0	3	3
Fusion	0	3	3	3	4	1	2	1	2
Degenerative changes	0	0	0	0	1	1	1	0	2
Vasodilatation of secondary lamellae	0	0	1	1	2	0	0	1	2
Vasodilatation of main venous sinus	0	1	1	1	2	1	2	2	2

(0) – no histological alterations, which represented normal histological structure; (1) – mild histological alterations; (2) – moderate histological alterations; (3) – severe histological alterations; (4) – very severe histological alterations in the gill surface architecture

tive processes, which lead to a rapid increase in the thickness of the gill epithelium and fusion of the adjacent secondary lamellae (Fig. 1B).

The degenerative changes were found in a higher degree in the fish treated with Pb (Table 3, Fig. 1F). This implies necrotic processes due to the exposure to this heavy metal. In parallel with these processes, processes of cell division were included, which were expressed with the observed proliferative changes. Gradually, the degenerative changes displaced the proliferative ones, and therefore a mild degree of expression of fusion was found the fish exposed to lead. Such alterations are presented by Jabeen, Chaudhry (2010).

Similarly to Patnaik et al. (2010) we also found changes in the blood vessels of the gills, which tend to increase the degree of expression with the increasing of the heavy metal concentrations. In addition, Ni and Pb caused increase in the respiration rate, which in turn probably lead to an increased flow of red blood cells and increased inner pressure, resulting in vasodilation of the main venous sinus and secondary lamellae, which were more pronounced in the fish exposed to Pb (Table 3, Fig. 1C).

Conclusion

Overall, changes in the respiration rate and histological structure of the gills of common carp showed that serious disturbances in the physiological and cellular metabolism under the influence of Ni and Pb occurred. These changes probably allow the fish to activate their adaptive and protective mechanisms. Respiration rate measurements and histological alterations can be applied as sensitive biomarkers for Ni and Pb contamination in biomonitoring programs in order to assess the impact in freshwater ecosystems.

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