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Exposure of silver carp (*Hypophthalmichthys molitrix*) to environmentally relevant levels of cadmium: hematology, muscle physiology, and implications for stock enhancement in the Xiangjiang River (Hunan, China)

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Cadmium is a non-essential metal with a wide distribution that has severe toxic effects on aquatic animals. Changes in hematology and muscle physiology were examined in silver carp (*Hypophthalmichthys molitrix*) exposed to environmentally relevant levels of cadmium (0.01 mg L^{-1}) for 96 h. Cadmium exposure induced significant increases in the red blood cell count, and in the plasma concentrations of cortisol, glucose, and lactate. This suggests that the dose of cadmium was sufficient to cause stress, possibly associated with impaired gas exchange at the gills. There were no changes in hemoglobin concentration or plasma protein concentration. Significant decreases in muscle energy fuels (ATP and glycogen), and increases in muscle lactate persisted until the end of the exposure period, respectively. The changes in muscle lactate and protein in silver carp differed from those observed in response to exposure of fish to cadmium and heavy metals in other studies. The study highlights the importance of selecting unpolluted release sites with suitable water conditions for the survival of newly released individuals for stock enhancement of the Xiangjiang River.

silver carp, hematology, muscle physiology, cadmium exposure, stock enhancement

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For decades, stock enhancement has been used to manage severely exploited recruitment-limited fisheries around the world [1,2]. However, for a number of reasons, wild populations have not always shown signs of recovery [3]. The primary factor appears to the stress response and consequent higher mortality of the released individuals, particularly in the period immediately following release. Therefore, it is important to develop techniques to minimize this early post-release mortality [4,5].

Historically, the Xiangjiang River was important because the Hengyang section provides spawning habitats for the four major Chinese carps that occur downstream in the middle reaches of the Yangtze River. The four major Chinese carps have been in severe decline because of the construction of hydraulic projects, over-fishing, sand excavation, and water pollution [6]. Therefore, to maintain the fish resource, stock enhancement has been carried out in Hunan Province since 2003 [7]. According to official statistics, nearly 20000 fish were released during 2005–2007, but the total was probably much higher because of escapes during the rainy seasons. While much attention has been focused on the number of fish released, techniques of release have

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received little consideration, and survival after release has seldom been systematically estimated [7]. Furthermore, the Xiangjiang River is subjected to heavy metal pollution [8], and concentrations of cadmium (Cd) are especially high. Over several years, mean values of Cd concentration have varied from below 0.005 to 0.431 mg L^{-1} [9,10]. Although it is expected that heavy-metals pollution would adversely affect fish populations [11], there has been little experimental investigation of the relationship between the levels of heavy metals that occur in the Xiangjiang River and the survival of the newly released individuals.

Exposure of fish to heavy metals could have widespread detrimental effects on their health because of metabolic requirements of detoxification and repair mechanisms [12,13]. The adverse effects of environmental contaminants (including heavy metals) on fish are manifested at various levels of organization [14,15]. Biochemical and cellular responses include elevated levels of blood glucose and lactate, decreased levels of glycogen, ATP and phosphocreatine (PCr) in tissues, and changes in hematocrit and hemoglobin [16,17]. It has been suggested that these responses could be used as early warning signs of exposure to toxic chemicals because biochemical and cellular changes must precede detectable effects at level of the individual organism (e.g., behavior), population or community [18,19]. Thus, estimation of responses to heavy metals may provide sensitive indicators on which to predict the effects of heavy-metal pollution on fish populations.

Cadmium is a non-essential metal with a wide distribution and is one of the most toxic heavy metals [20–23]. Even short-term pollution of water with cadmium may result in severe physiological disturbances to fish that develop and persist when the metal is no longer present in the water [24]. Previous studies have mainly concentrated on the effects of waterborne cadmium on the anti-oxidative systems of aquatic animals; the specific effects of cadmium on the muscle energy stores in fish have received less attention. In particular, there are few reports on the effects of cadmium and other heavy metals on filter-feeding fish such as silver carp. This information will broaden the knowledge about the toxic effects of heavy metals on aquatic animals.

The objective of this study was to examine the effects of cadmium at levels found in the Xiangjiang River on blood and muscle characteristics of silver carp (*Hypophthalmichthys molitrix*). This information will help to evaluate the potential effects of cadmium pollution in the river, advance knowledge on the effects of heavy metals on filter-feeding fish, provide biological information relevant to stock enhancement. It has been shown that low levels of cadmium (0.01 mg L^{-1}) stimulate increased activities of superoxide dismutase and catalase in serum, liver, gills, and muscles of fish [15]. It was hypothesized that similar levels of cadmium disturb the hematology and muscle energy stores of silver carp.

1 Materials and methods

1.1 Animal holding

Silver carp were obtained from the National Original Breeding Farm in Changsha, China. All fish were kept in a rectangular rearing pond (length×width×water depth: 22 m×17 m×1.2 m) with abundant phytoplankton, at the Hunan Agricultural University, and were exposed to seasonal temperatures. The phytoplankton community was dominated by *Microcystis aeruginosa*, *Anabaena circinalis*, *Crucigenia apiculata*, *Scenedesmus quadricauda*, *Cryptomonas ovata*, and *Synedra acus*. The fish were held for at least two months before the experiment.

1.2 Cadmium exposure

Sixty-four size-matched fish were selected from the holding pond. To eliminate differential effects of prior feeding, they were transferred to a fiber glass holding tank (diameter 100 cm; depth 50 cm) supplied with aerated and dechlorinated tap water flowing at 3 L min^{-1} before cadmium exposure. After 24 h evacuation, the pre-selected fish were randomly distributed into 64 circular fiberglass tanks (water volume 52 L). The fish were acclimated to aerated deionized water and allowed to recover from handling stress for 12 h. Cadmium exposure was initiated by adding concentrated cadmium chloride solution (0.5282 mg mL^{-1}, pH 7.2, $CdCl_2 \cdot 2.5\ H_2O$, Sinopharm Chemical Reagent, Co., Ltd., Shanghai, China) to each holding tank, except for the control tanks. The solution was introduced through a hole in the lid to minimize disturbance to the acclimated fish. The nominal concentration (0.01 mg L^{-1}) is environmentally relevant since it is the quality standard for surface water and a level frequently found in the Xiangjiang River, especially in the lower reaches near large industrial cities such as Zhuzhou and Xiangtan. Blood and muscle samples were obtained after continuous exposure for 0, 1, 6, 12, 24, 48, 72, and 96 h. At each sampling time, eight fish were randomly selected and sedated in their tanks by adding 2 mL of clove oil (1.04 g mL^{-1}, Sinopharm Chemical Reagent, Co., Ltd., Shanghai, China) through the hole in the lid. This ensured that all fish were sedated within 1 min time without noticeable struggling. Water samples prepared for cadmium determination were taken immediately after completion of the fish sampling. Total cadmium concentrations in the water, as measured by atomic absorption spectroscopy (SP-3803, Spectrum Shanghai, China), ranged between 0.009 and 0.012 mg L^{-1} and significant differences among seven exposure groups were not detected (P>0.05). During the exposure period, dissolved oxygen was >7 mg L^{-1}, ammonia nitrogen was <0.015 mg L^{-1}, and the pH ranged from 7.0 to 7.3. The average water temperature was 5.9±0.2°C. The photoperiod was controlled by artificial lighting (illuminated from 08:00 to 18:00).

1.3 Sampling protocols

When gill irrigation had ceased, the fish were removed from the holding tank and blotted of excess water; their weights were measured to the nearest 0.1 g, and standard lengths to 0.1 cm. Blood samples (about 1.0 mL) were taken from the caudal vessel using 2 mL heparinized syringes. The fish were then immediately placed on ice and slices of muscle tissue (about 8 g) were removed from between the anterior insertion of the dorsal fin and the lateral line, freeze clamped in liquid nitrogen, and stored at −80°C until later analysis. An aliquot of each blood sample was used to determine the red blood cell (RBC) count and hemoglobin content. The remainder was centrifuged at 2600×*g* for 5 min at 4°C to collect the plasma, which was stored at −80°C for later measurement of glucose, lactate, total protein, and cortisol concentrations.

1.4 Analytical techniques

Blood samples were diluted 200-fold with 0.85% NaCl and the RBC count was determined using a Neubauer hemocytometer under a microscope. Total blood hemoglobin (Hb) concentrations were estimated spectrophotometrically at 540 nm using the cyanmethemoglobin method (Maker Technology Co., Ltd., Chengdu, China). Plasma protein concentration (Bradford method), plasma glucose (Glucose oxidase-peroxidase method), plasma lactate (colorimetry), plasma cortisol (ELISA method), muscle protein (Bradford method), muscle lactate (colorimetry), muscle glycogen (anthrone colorimetry), and muscle ATP (phosphomolybdate colorimetry), were all measured using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

1.5 Statistical analysis

All values are presented as means±SE and all data were tested for normality using Shapiro-Wilk's W test and for homogeneity of variances using Levene's test. When necessary, log10-transformations were performed. Differences among exposure groups in fish weight and length were analyzed using one-way ANOVA. Physiological parameters at each sample time were statistically compared with values for the control group using one-way ANOVA and Dunnett's test. All analyses were carried out using Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA), with $P<0.05$ considered statistically significant. The figures were created using Origin 6.1 software (OriginLab Corp., Northampton, MA, USA).

2 Results

2.1 Blood parameters

The mean body weight and mean standard length of the experimental fish were (105.4±0.9) g and (18.2±0.1) cm, respectively, and did not differ significantly among the different exposure groups ($P>0.05$). The mean RBC count of control fish was 1.50×10^6 mL^{-1}. Following Cd exposure, the RBC count increased sharply in the first hour and was elevated by 46% after 12 h. Mean RBC count then gradually declined, but it remained higher than the control value until the end of exposure period (significantly different from control RBC count only at 72 h; Figure 1A). Exposure to cadmium did not significantly affect Hb or plasma protein concentrations (overall mean values: (71.72±2.14) and (20.69±0.52) g L^{-1}; $n=64$; $P>0.05$) (Figure 1B and 1F).

The mean plasma cortisol concentration of the non-exposed control group was (297.76±4.11) ng L^{-1}. Plasma cortisol concentration increased steadily during the Cd exposure period. It was significantly higher than controls values after 24 h and was maximal at the end of the 96 h exposure period ((527.51±21.50) ng L^{-1}) (Figure 1C). The mean plasma glucose concentration of control fish was (2.71±0.15) mmol L^{-1}. Exposure to cadmium elicited a significant increase in plasma glucose concentration with peaks at 12 h ((3.95±0.16) mmol L^{-1}) and 96 h ((3.81±0.44) mmol L^{-1}). Mean plasma glucose was elevated throughout the exposure period (significantly different at 1, 12, and 96 h; Figure 1D). The mean plasma lactate concentration of control fish was (2.94±0.25) mmol L^{-1}. The lactate concentration of the plasma dipped slightly after 6 h Cd exposure and then increased steadily to a maximum value of (4.72±0.31) mmol L^{-1} at the end of exposure period (but significantly different from controls at 72 and 96 h; Figure 1E).

2.2 Muscle parameters

The mean ATP concentration in the muscle of control fish was (135.9±11.6) μmol g^{-1}. Cadmium exposure steadily depleted the muscle ATP with a marked decrease to about half of the control value between 24 and 48 h (significant at 48, 72, and 96 h; Figure 2A). The mean muscle glycogen was (2.35±0.17) mg g^{-1} for control fish. It decreased significantly after 1 h exposure and continued to decline until the end of exposure period, at which the lowest value was reached ((0.75±0.10) mg g^{-1}; Figure 2B).

The mean lactate concentration in the muscle of control fish was (9.56±0.93) μmol g^{-1}. Lactate decreased sharply and significantly after 1 h exposure, returned to close to the control level after 6 h, and then significantly decreased again to its lowest value after 12 h ((5.06±0.23) μmol g^{-1}). Thereafter, muscle lactate progressively increased with the exposure time to a value significantly elevated above the control value at the end of the exposure period ((14.44±0.73) μmol g^{-1}; Figure 2C). The mean protein concentration in the muscle of control fish was (78.82±4.08) mg g^{-1}. It increased significantly with Cd exposure time, peaking at 12 h

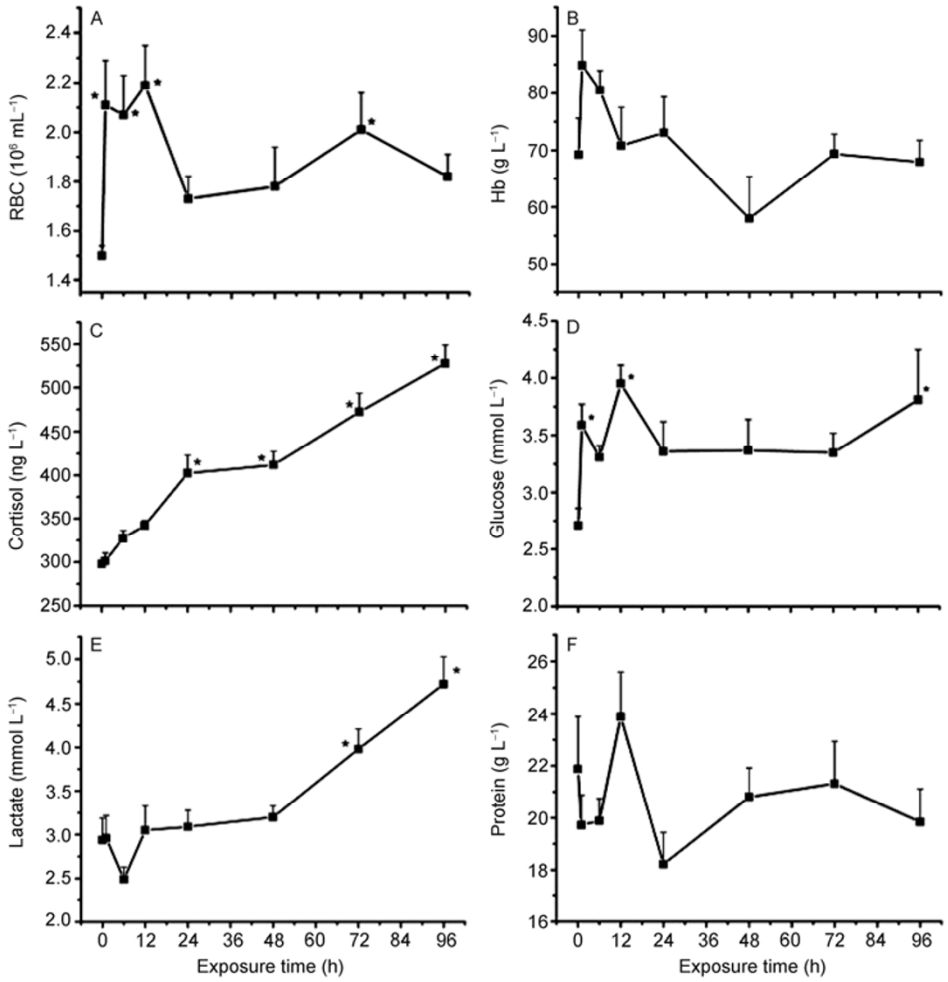

Figure 1 Time course of changes in blood and plasma constituents in silver carp exposed to sublethal Cd concentrations in the water. A, Red blood cell count. B, Hemoglobin concentration (Hb). C, Cortisol concentration. D, Glucose concentration. E, Lactate concentration. F, Protein concentration. Values are mean±SE (n=8). An asterisk (*) denotes a significant difference from the non-exposed control group; P<0.05.

((107.43±7.12) mg g^{-1}) and then plateauing except for a small decrease at 72 h (Figure 2D).

3 Discussion

The present study supported the hypothesis that even relatively low levels of cadmium (0.01 mg L^{-1}) are able to disturb the hematology and muscle energy stores of silver carp. The dose (0.01 mg L^{-1}) is considered a sublethal concentration, since it was approximately 1/280th of the acute toxicity value (LC_{50}) for silver carp fingerlings (about 6.5 cm body length) [25]. Accordingly, no mortality was recorded in the cadmium-exposed silver carp.

3.1 Hematological responses to cadmium exposure

Exposure of silver carp to cadmium elicited a significant increase in plasma cortisol. Plasma cortisol is considered to be a sensitive indicator of stress in fish [16,17,26], which implies that the exposed carp were in a stressed state. Similar increases in plasma cortisol have been reported in other fish exposed to cadmium, and to other heavy metals, but the changes generally occurred within 4 h [27]. In silver carp, a significant increase in cortisol was observed only after 24 h exposure, although mean values began to increase immediately. The slow rise of plasma cortisol may be a reflection of the low level of exposure to Cd. It is noteworthy that the circulating plasma glucose had increased after only 1 h of exposure (Figure 1D). These observations are consistent with suggestions that high levels of circulating glucose in response to stressor are sustained by increased plasma cortisol after an initial catecholamine-induced increase [16,17]. Increased lactate levels after heavy metal exposure are considered to result from anaerobic metabolism caused by gill impairment [28]. In the present study, plasma lactate was significantly elevated after 72 h exposure. This suggests that, in silver carp, gas exchange at the gills was damaged after 72 h of exposure to 0.01 mg L^{-1} cadmium. However, histological analysis of the gill should be performed to verify this

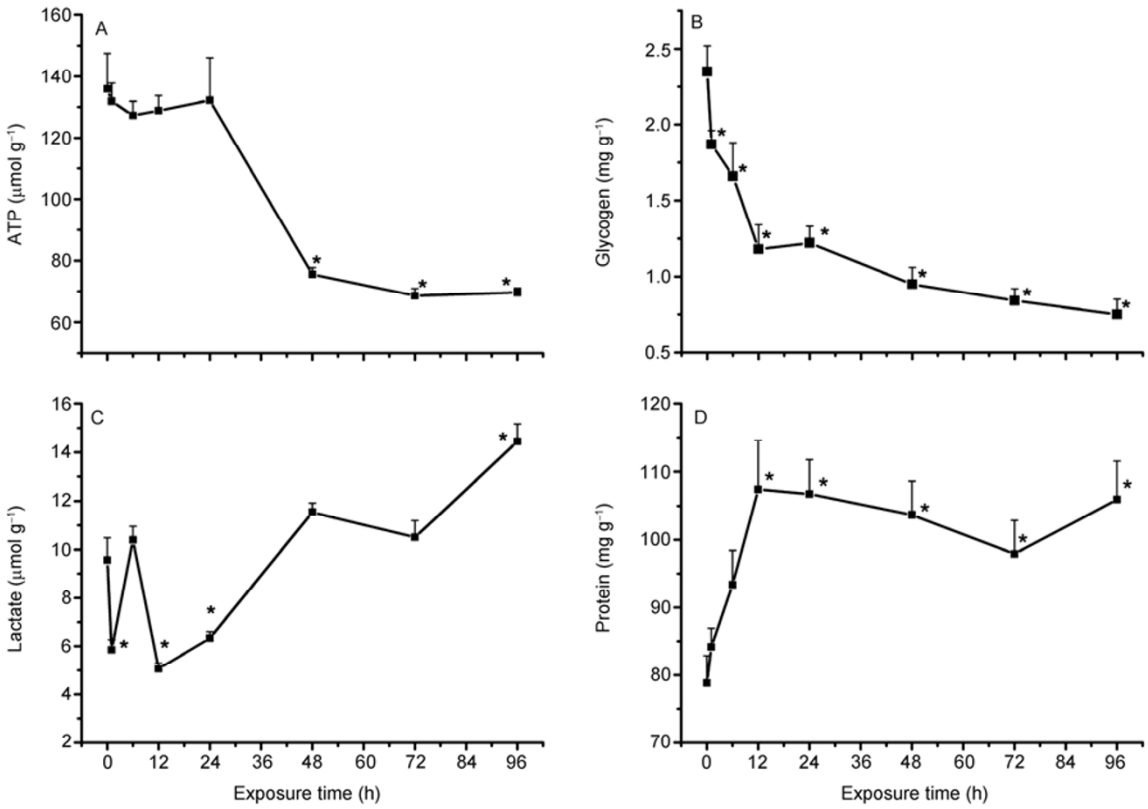

Figure 2 Time course of changes in muscle constituents in silver carp exposed to sublethal Cd concentrations in the water. A, ATP concentration. B, Glycogen concentration. C, Lactate concentration. D, Protein concentration. Values are mean±SE (n=8). An asterisk (*) denotes a significant difference from the non-exposed control group; P<0.05.

postulation. Cadmium exposure elicited enhanced erythropoiesis, as indicated by the gradual increase in RBC count. Presumably, this was induced by hypoxia resulting from impaired gas exchange at the gills, as has been reported in other fishes [29]. There were no clear changes in plasma Hb and protein, possibly because these secondary responses to environmental stress depend upon the exposure scenario and the acclimatization condition of fish [16,17,30]. Similar results have been reported on other fishes [31–33].

3.2 Muscle responses to cadmium exposure

Muscle glycogen and ATP decreased immediately after exposure to cadmium. Glycogen depletion persisted until the end of the exposure period, when the lowest values were reached (Figure 2B). A similar response was reported in common carp exposed to sublethal copper, where depletion of muscle glycogen persisted for three weeks [34]. The response pattern of muscle ATP was similar to that of muscle glycogen. At the end of the exposure period, the muscle ATP had decreased to its lowest value at only 49% of that of controls (Figure 2A). Depletion of muscle energy fuels suggests that the silver carp exposed to cadmium invested the stored energy in detoxification and repair mechanisms [13]. There have been no previous studies on the effects of Cd or heavy metals on muscle energy stores in silver carp or other filter-feeding fish. Previous studies have shown that depletion of muscle glycogen due to anaerobic glycogenolysis resulted in accumulation of muscle lactate [35–37]. Surprisingly, muscle lactate decreased during the first 24 h of cadmium exposure. Thereafter, muscle lactate increased again and was significantly elevated above the control level at the end of the exposure period (Figure 2C). As plasma lactate did not increase until 72 h of exposure when muscle lactate had recovered to the control level and had begun to accumulate, it is unlikely that the initial decrease in muscle lactate was caused by the leakage into the blood space. Significant decreases of muscle lactate were also observed in common carp (*Cyprinus carpio*) after 3 and 7 days of exposure to copper, where it was speculated that muscle lactate was used as an energy source in aerobic metabolism [38]. In addition, previous studies have shown that muscle glycogen resynthesis occurred *in situ* using lactate as the primary substrate. This process could involve direct uptake of lactate from the blood space by the muscle [37]. Therefore, it is possible that silver carp use muscle and plasma lactate for glycogen resynthesis to meet the extra need of energy for detoxification and repair. It is difficult to understand the decrease of plasma lactate and increase in muscle lactate observed after 6 h exposure. Possibly, the drop in

plasma lactate contributed to the increase of muscle lactate but further research is needed to determine the exact mechanism.

Cadmium exposure elicited a significant increase in muscle protein. This effect was pronounced after 12 h exposure and persisted until the end of experiment (Figure 2D). This is an interesting observation because it conflicts with other reports of the pattern of secondary responses to stressors (including to cadmium); generally protein synthesis was inhibited and muscle protein concentration decreased [16,17,34], or no change depending on organs or muscle type, and so forth [39–41]. Further studies should explore this interesting physiological process. Possibly, changes in protein concentration reflect modifications of muscle water content resulting from impaired osmoregulatory function of the gills [42,43].

3.3 Implications for stock enhancement in the Xiangjiang River

Many previous studies have demonstrated that the swimming performance of fish is impaired by metal exposure, including rainbow trout (*Oncorhynchus mykiss*) [44–46], brown trout (*Salmo trutta*) [47–49], common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*) [38]. This has been attributed to depletion of muscle energy fuels (PCr, ATP and glycogen) and accumulation of metabolic end products (lactate and ammonia) [50]. The present study has shown that low level (0.01 mg L^{-1}) cadmium exposure enhanced erythropoiesis, increased plasma levels of cortisol, glucose and lactate, decreased muscle glycogen and ATP, and increased muscle protein and lactate. This implies that 0.01 mg L^{-1} cadmium would impair the swimming abilities of silver carp, although further studies should be carried out to confirm this assertion. Swimming performance is considered a primary characteristic determining the survival of many species of fish [51–53]. Therefore, these changes could have profound biological significance in relation to stock enhancement in the Xiangjiang River, in which cadmium concentrations of the lower reaches often exceed 0.01 mg L^{-1}. Release sites for silver carp should be established in the upper reaches to eliminate the effects of heavy metal pollution on survival of newly released individuals. In addition, the stock enhancement programs in the lower reaches should be initiated during the rainy seasons when the heavy metal levels are low. In the long term, effective restoration and maintenance of the fish resources of the Xiangjiang River, will involve simultaneous fish recovery programs and remediation of heavy metals pollution.

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