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STUDIES ON THE TOXIC EFFECTS OF LEAD AND NICKEL MIXTURE ON TWO FRESHWATER FISHES, *CTENOPHARYNGODON IDELLA* AND *HYPOPHTHALMICHTHYS MOLITRIX*

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ABSTRACT

The Probit Analyses was used to determine the acute toxicity of binary mixture of lead and nickel for two fish species viz. grass carp (*Ctenopharyngodon idella*) and silver carp (*Hypophthalmichthys molitrix*). Correlation and regression analyses were also computed to find-out relationships among various parameters. The criteria of toxicity used during these experiments were mortality upon two fish species for 90-day. The tests were performed separately at constant pH (7), temperature (30°C) and hardness (200 mgL^{-1}) of water with three replications for each test dose. There existed significant differences for LC_{50} and lethal responses for both grass carp and silver carp. The mean LC_{50} and lethal concentrations for grass carp were 56.42±2.51 and 120.98±7.18 while that of silver carp the same were 55.85±2.84 and 128.44±9.25 mgL^{-1}, respectively. Among two fish species silver carp was found significantly more sensitive in terms of 96-hr LC_{50} than that of grass carp. However, grass carp appeared significantly more sensitive than silver carp for lethal responses. The metallic ion concentrations of test mediums showed positively significant correlation with carbon-dioxide, sodium, potassium and electrical conductivity while inverse relationship was identified for dissolved oxygen for both fish species in the test media.

Key words: Acute Toxicity, Metal Mixture, Lead, Nickel, Grass carp, Silver carp.

INTRODUCTION

The environmental conditions are not static and human influence has greatly stimulated the flow of environmentally deleterious changes by loading with chemicals to the aquatic system. The heavy metal and pesticide contamination of aquatic system has attracted the attention of researchers all over the world (Javed, 2012). Heavy metals have been recognized as serious pollutants of the aquatic environment that are causing serious impairment in metabolic, physiological and structural systems of both animals and plants. The accumulation of metals in an aquatic environment has direct impacts on the sustainability of the ecosystem. Interest in the heavy metals, which are required for metabolic activities in organisms, lies in the narrow range between their essentiality and toxicity (Jabeen, 2012). Municipal wastewater and industrial effluents used to irrigate agricultural land contain massive quantities of different heavy metals (Ashfaq *et al*., 2012) which usually penetrate and concentrated in different tissues of fish body viz. skin, gills and intestine through water and food (Ahmad and Bibi, 2010). Fishes are considered as one of the most significant indicators in freshwater systems for the estimation of metal pollution (Azmat *et al.,* 2012). The commercial and edible fish species have been widely investigated in order to check heavy metal contamination particularly those that are hazardous to human health. With the growth of fishes, the heavy metals accumulate in the internal organs like muscles, liver and intestine in a considerably higher concentration, make the fish unsuitable for human consumption (Ahmad *et al.,* 2011).

During acute toxicity tests, juvenile fish (*Catla Catla*, *Labeo rohita* and *Cirrhina mrigala*) were exposed to wide range of toxicant concentrations in a static water system for 96-hr. A toxic effect was determined by a statistically significant decrease in the survival rate of fish exposed to a particular metal, relative to survival of fish in control (Yaqub and Javed, 2012).

Most of the lead in the environment is in inorganic form, exists in multiple oxidation states (Jackson *et al*., 2005). Sublethal concentrations of lead can result in the decreasing trend of feeding energy, growth rate and biochemical composition of fish (Kasthuri and Chandran, 1997). Nickel is a transition metal, and is generally considered an important micronutrient, always found at a low level in animal tissues and seems to be well regulated. Excess and deficiency of nickel depicted negative impacts on fish survival rate (Eisler, 1998). Many studies concerning harmful impacts of metals on fish are associated with individual metals whereas the aquatic organisms are generally exposed to metals mixtures. Some metals can alter the accumulation of the other metals in the body of fish (Jezierska and Sarnowski, 2002).

In the Punjab province (Pakistan), the natural freshwater streams have been polluted drastically with heavy metals as a result of bulk discharges of sewage water and unprocessed industrial toxins that is significantly distressing the fish fauna. Therefore, to facilitate the conservation of freshwater fisheries in the natural waters of Punjab province, it is pertinent to reveal the tolerance limits of cyprinids against metals mixtures. This will help to plan policies for suitable conservation of fish species and to predict possible effects of persistent metal's pollution in the aquatic habitats of Pakistan.

MATERIALS AND METHODS

The present study was conducted in the laboratory of Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. An acute toxicity of lead and nickel mixture (binary mixture in equal proportion) to fish, grass carp and silver carp, were determined in terms of 96-hr LC_{50} and lethal concentrations at constant pH (7), temperature (30°C) and hardness (200 mgL^{-1}) of water. Fishes were acclimated to laboratory conditions prior to start of experiment. Fishes were fed twice a day with crumbled feed (30 % Digestible Protein and 2.70 Kcal g^{-1} Digestible Energy) during adaptation, but they were not fed during last 24 hours of adaptations and throughout the test duration. Leftover feed and feacal wastes were removed from all the aquaria through vacuum pumps. These tests were performed in glass aquaria each having volume of 70 liters de-chlorinated tap water. After acclimation to laboratory conditions the fish were transferred to the glass aquaria for toxicity tests. Chemically pure compounds of lead ($PbCl_2$) and nickel ($NiCl_2.6H_2O$) were dissolved as per desired weight in deionized water and stock solutions were prepared for required metal mixture (Pb+Ni) dilutions starting from 0.00 to 120.00 mgL^{-1}. Average weight and total lengths of fish used in this experiment are given in Table 1. Ten (10) fish of each species were kept in each glass aquarium with three replicates for each test dose. In order to eliminate stress to fishes, the concentration of metal mixture in each aquarium was increased gradually and 50 percent test concentration was maintained within 3.5 hours and full toxicant concentration in 7 hours. Constant air was supplied to all aquaria. The metal concentration for each fish species was started from zero with an increment of 0.05 and 5 mgL^{-1} (as total concentration) for low and high concentrations, respectively. During each test trial, the observations on fish mortality and physico-chemical variables viz. temperature, pH, total hardness, dissolved oxygen, total ammonia, sodium, potassium and carbon-dioxide were made at 12 hours intervals during 96 hour (APHA, 1998). The data on different variables, obtained from this experiment were statistically analyzed through Micro-Computer by following Steel *et al.* (1996). Analysis of Variance (Factorial Experiment) and Duncan's Multiple Range tests were performed to find-out statistical differences among variables under study. Correlation analyses were also performed to find-out relationships among various parameters under study. The data on percent fish mortality, obtained during 96 hour LC_{50} and lethal concentration trials were analyzed by using probit analysis method (Hamilton *et al*., 1977).

RESULTS

The acute toxicity (96-hr LC_{50} and lethal concentrations) were used to evaluate the sensitivity of two fish species viz. grass carp and silver carp against binary mixture of two metals. Grass carp and silver carp, with average weights of 3.21±0.11 and 2.64±0.11g, respectively, were tested, separately, for their sensitivity towards lead and nickel (combine form) as 96-hour LC_{50} and lethal responses with 95% confidence interval.

Acute Toxicity of Metal Mixture to the Fish: The data on sensitivity of grass carp and silver carp to short term exposure of lead and nickel mixture determined as their 96-hour LC_{50} and lethal responses with 95% confidence interval are presented in Table 2. Fishes were exposed to various concentrations of metal mixture to determine their 96-hour LC_{50} and lethal responses. Both fish species showed variable sensitivity to metal mixture at different set of variables. Grass carp showed mean 96-hour LC_{50} of 56.42±2.51 mg L^{-1} with confidence interval range of 51.45–61.52 mgL^{-1} while its mean lethal concentration was computed as 120.98±7.18 mgL^{-1} with the confidence interval of 109.09–138.35 mgL^{-1}. The regression coefficient computed for the probability graph was highly significant exhibiting the fillness of this regression line. This model reveals Deviance Chi-square value of 5.832 and goodness of fit test p as 0.924 that showed high precision of this regression line (Figure 1). Mortality data obtained during acute toxicity of metal mixture tests with silver carp are also presented in Table 2. Normal distribution of data has been estimated at 95% confidence interval, showing LC_{50} value of 55.85±2.84 mgL^{-1} with confidence interval range of 50.37-61.81 mgL^{-1} while the mean lethal concentration of metal mixture for this fish was computed as 128.44±9.25 mgL^{-1} with confidence intervals of 113.47-151.57 mgL^{-1}. The Deviance Chi-square value of this line was computed as 7.825 and goodness of fit test p as 0.729 (Figure 2). It is evident from Table 2 that there existed non-significant differences among three replicates mediums used for both grass carp and silver carp, separately, to determine their mean LC_{50} and lethal concentrations of metal mixture. However, there existed significant differences between metal mixture LC_{50} and lethal concentrations for both fish species. Under all test trails with constant temperature, pH and hardness of water, silver carp exhibited significantly more sensitive to binary mixture of lead and nickel in terms of 96-hr LC_{50} than that of grass carp.

However, grass carp appeared significantly more sensitive than silver carp for lethal responses.

Table 1. Average weights and lengths of fish species during acute toxicity trails.

Fish Age (Days)	Fish species	Wet weight (g±SD)	Total length (mm±SD)
90-day	Grass carp	3.21±0.11	55.88±0.36
	Silver carp	2.64±0.11	51.21±0.09

Table 2. Percent (%) mortality of fish during acute exposure of Pb+Ni mixture for 96 hours.

Fish Age	Exposure concentrations (mgL^{-1})	Fish Mortality (%)							
		Ctenopharyngodon idella				*Hypophthalmichthys molitrix*			
		R_1	R_2	R_3	Mean	R_1	R_2	R_3	Mean
	5	0	0	0	0.00	0	0	0	0.00
	10	0	0	0	0.00	10	10	10	10.00
	15	10	10	10	10.00	10	20	20	16.67
	20	10	20	20	16.67	20	20	20	20.00
	30	20	20	20	20.00	30	30	30	30.00
90-day	40	30	30	30	30.00	30	40	40	36.67
	50	30	40	40	36.67	40	40	35	40.00
	60	40	40	40	40.00	50	50	40	46.67
	65	50	50	50	50.00	50	50	50	50.00
	70	60	60	60	60.00	60	60	60	60.00
	75	70	70	70	70.00	80	70	70	73.33
	80	80	80	80	80.00	90	80	90	86.67
	90	90	90	90	90.00	100	100	100	100.00
	100	100	100	100	100.00	-	-	-	-

Fish Species	Mean Metal Mixture Concentration (mgL^{-1} ±SD)	
	Ctenopharyngodon idella	*Hypophthalmichthys molitrix*
LC_{50}	$56.42±2.51^a$	$55.85±2.84^b$
Lethal Concentration	$120.98±7.18^b$	$128.44±9.25^a$

The mean with different letters in a single row are statistically different at p< 0.05.

Normal Distribution (Maximum Likelihood Estimates at 95% Confidence Interval)

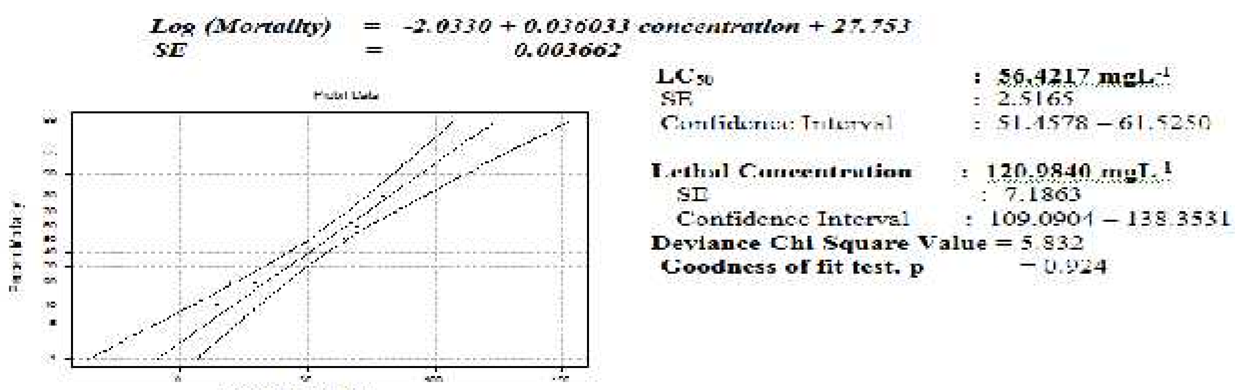

Figure 1. Probability graph for 96-hr LC_{50} and lethal concentrations (mgL^{-1}) determined for *Ctenopharyngodon idella*

Water Quality Characteristics: Data regarding correlation coefficients among different physcio-chemical variables of the test mediums and the metal mixture exposure concentrations used for grass carp and silver carp are presented in Table 3. The metallic ion concentrations of the both test mediums showed positively significant relationships with total ammonia, carbon-dioxide, electrical conductivity, sodium, potassium while inverse relationship with dissolved oxygen of the test media. There was positively significant correlation among carbon-dioxide, electrical conductivity, sodium, potassium for ammonia while the same was negative but significant with dissolved oxygen for both fish species. Dissolved oxygen exhibited inverse relationship with carbon-dioxide, electrical conductivity, sodium, and potassium while that of the carbon-dioxide was positively significant with electrical conductivity, sodium, potassium. Electrical conductivity had positively significant relationship sodium, potassium while sodium exhibited positively significant correlation with potassium during both media used for Grass carp and silver carp. Calcium showed positively significant relationship with magnesium for silver carp while that between potassium and calcium was negative but significant at p<0.05 of test media used for acute toxicity tests for grass carp.

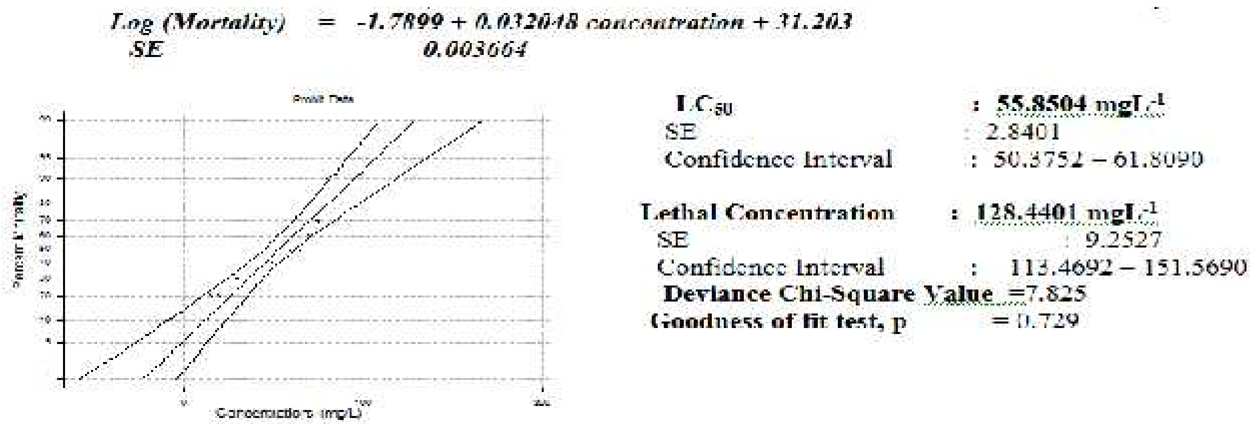

Figure 2. Probability graph for 96-hr LC_{50} and lethal concentrations (mgL^{-1}) determined for *Hypophthalmichthys molitrix*. Normal Distribution (Maximum Likelihood Estimates at 95% Confidence Interval)

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Table 3. Correlation coefficients among various physico-chemical parameters of the test media (water).

	Conc.	Temp	pH	T.H.	$T.NH_3$.	DO	CO_2	E.C.	Na	K	Ca
Ctenopharyngodon idella											
Temp.	0.27599										
pH	0.26071	0.13754									
T.H.	0.24964	-0.16935	0.27355								
$T.NH_3$.	0.83020	0.20470	0.23124	-0.19817							
D.O	-0.74936	-0.14610	-0.17896	0.17527	-0.98641						
CO_2	0.96290	0.25376	0.26101	-.21479	0.94182	-0.89255					
EC	0.94788	0.31084	0.16057	-0.23361	0.84381	-0.79901	0.95332				
Na	0.95372	0.25605	0.23870	-0.24020	0.95188	-0.90676	0.99802	0.95172			
K	0.95375	0.36664	0.26002	-0.23569	0.89532	-0.84647	0.97243	0.98141	0.96964		
Ca	0.47318	0.52444	-0.28162	-0.30961	0.37767	-0.34169	0.47043	0.55020	0.47002	0.53663	
Mg	-0.06696	-0.75917	-0.19704	-0.7931	-0.09568	0.09430	-0.08694	-0.13095	-0.08451	-0.20435	-0.37439
(Critical Value (2 tail 0.05) ± .53067)											
Hypophthalmichthys molitrix											
Temp	-0.14574										
pH	0.35783	0.46527									
T.H.	-0.16315	0.21077	0.10390								
$T.NH_3$.	0.84137	-0.15364	0.28141	-0.16924							
D.O	-0.85859	0.14597	-0.25265	0.12925	-0.98581						
CO_2	0.96664	-017147	0.30910	-0.16895	0.94909	-0.95890					
EC	0.90648	-0.44788	0.16077	-0.28970	0.79516	-0.78647	0.89377				
Na	0.931446	-0.17582	0.31562	-0.13725	0.92780	-0.94349	0.97129	0.87932			
K	0.94303	-0.21319	0.30974	-0.11568	0.87993	-0.91105	0.95838	0.86755	0.98339		
Ca	0.24174	-0.22263	-0.21417	0.22619	0.28098	-0.33750	0.28863	0.23233	0.41796	0.42416	
Mg	-0.24933	0.25764	0.23596	-0.13949	-0.29674	0.34235	-0.29879	-0.26673	-0.42076	-0.42328	0.98892
(Critical Value (2 tail 0.05) ± .51235)											

Conc. =Concentration(mg L^{-1}); Temp = Temperature (^{O}C); T.H. = Total hardness (mg L^{-1}); $T.NH_3$=Total ammonia (mg L^{-1}); D.O = Dissolved oxygen (mg L^{-1}); CO_2 =Carbon Dioxide; E.C. = Electrical Conductivity (mS cm^{-1}); Na = Sodium (mg L^{-1}); K = Potassium (mg L^{-1}); Ca = Calcium (mg L^{-1}); Mg = Magnesium (mg L^{-1}).

DISCUSSION

In Pakistan, the advancement in agriculture and industrial sectors have resulted into aquatic pollution due to discharges of untreated industrial wastes and runoff water into the water bodies causing devastating effects on the fish health (Javed, 2006). Majority of research work has been conducted on the toxic effects of single metal species. However, in natural waters organisms are typically exposed to the mixtures of metals. Therefore, in order to provide data supporting the usefulness of freshwater fish as indicator of heavy metal's pollution, the acute toxicity of mixture of metals has been determined for two freshwater fish species viz. Grass carp and silver carp.

The present investigation revealed that two fish species showed highly significant differences for their tolerances limits (determined ac LC_{50} and lethal responses) for the binary mixture of lead and nickel. 96-hr LC_{50} concentrations of metal mixture also varied significantly between two fish species. Grass carp appeared as species that showed significantly higher 96-hr LC_{50} than that of silver carp. However, the lethal concentrations of these two fish species were also different. These results are in confirmatory with the result of Naz and Javed (2012). They worked on three fish species (*Catla catla*, *Labeo rohita* and *Cirrhina mrigala*) under acute exposure of 19 mixtures of lead, nickel, iron, manganese and zinc. The result showed that all the three fish species showed significantly variable tolerance limits, in terms of 96-hr LC_{50}, against mixture of five metals. Regarding overall sensitivity of five fish species, *Labeo rohita* were significantly least sensitive. The behavioral changes, such as loss of equilibrium, shoaling nature, swimming with their bellies upwards as well as irregular opercular movement were noted. The acute toxicity of copper and zinc (singly and in mixture form) can affect the developmental stages of rainbow trout, *Oncorhynchus mykiss* (Kazlauskiene and Vosyliene, 2008). Acute toxicity of heavy metals (cadmium, zinc and copper) was determined individually or in mixture form for Chinese minnow (*Gobiocyprius rarus*) in its early developmental stages (embryos, larvae). The sensitivity of this fish to heavy metals was different that depends on exposure period and developmental stages. Mixtures of Cu+Cd and Cu+Zn showed synergistic lethal effects. The susceptibility of species of different phylogenetic positions and various developmental stages to toxicants has often been compared by using acute methods (Kai Sun *et al*., 1995). However, in nature many species have a direct impact of long-term exposure of lower concentration of toxicants or their mixtures. In vitro tests, the impact of sub-lethal toxicity on fish enabled us to analyze different functional changes in physiological systems of the species attributable to long-term exposure of toxicants (Kazlauskiene *et al*., 1999). The impacts of single metal on the fish have been mostly evaluated while studies on tolerance limits of fish to the metal mixture (more than three metals) are scarce (Kazlauskiene *et al*., 1996; Kazlauskiene and Burba, 1997). These studies have demonstrated that the effects of metal mixture differ in terms of toxicity on living organisms from the effect of single metal because toxicity of heavy metal mixture to the fish is dependent upon their concentration, duration of exposure and their specific composition (Vosyliene *et al*., 2003).

All aquatic organisms particularly fishes are directly and indirectly influenced by the physical characteristics of aquatic environment, especially the physico-chemical parameters of water (Gillis *et al*., 2008). The physico-chemical characteristics of metallic ion metal mixture exposure media, used during growth trials, exerted significant impacts on growth, condition factor, feed intake and feed conversion efficiency of fish viz. *Catla catla, Labeo rohita* and *Cirrinha mrigala* (Naz *et al.,* 2012). Acute metals toxicity of water-borne and dietary metals to the fish is influenced by various a-biotic environmental factors such as oxygen, hardness (Ghillebaert *et al*., 1995), pH and temperature (Kotze *et al*., 1999). In the media, there existed positive and significantly variable correlations of metal concentration with total ammonia, carbon-dioxide, electrical conductivity, sodium and potassium while the same was negatively significant with dissolved oxygen. The exposure of zinc, cadmium and their mixture have been reported to reduce the level of sodium, chloride and calcium in the serum of fish, *Oreochromis niloticus* (Firat and Kargin, 2010). Short and long term toxicity of metals can affect the Na^{+}, Mg^{+2}, K^{+} and Ca^{+2} in different tissues of fish, *Oreochromis niloticus*. Short-term exposure of metals appeared to be more toxic in altering these ionic levels in the tissues of fish than long term exposures (Atli and Canli, 2003). The correlation co-efficient between calcium and magnesium was also positively significant. These findings supported the work of Deleebeeck *et al.* (2006) who studied the effect of calcium, magnesium and pH on the toxicity of nickel on juvenile rainbow trout (*Oncorhynchus mykiss*) during exposure of 26 days. The chemical activities of calcium and magnesium reduced the nickel toxicity. Moiseenko and Kudryoavtseva (2001) evaluated the relationships of nickel with water chemistry, organs and fish tissues. Therefore, water chemistry has profound effects on the uptake and accumulation of metals that ultimately resulted in variable tolerance limits of two fish species as observed during present investigation.

The oxygen consumption by the fish decreased whereas ammonia increased significantly with the increase in the metal concentrations of the test media. Variations in oxygen consumption ratio have been reported as indicator of metals mixture toxicity to the fish, *Oncorhynchus mykiss* and *Cyprinus carpio*. (add reference here) The effect of metals on oxygen consumption by *Tilpia mossambica* was determined by Shereena and Logaswamy (2008). Significant decrease in the oxygen consumption by fish exposed to zinc sulphate, cobalt carbonate, lead nitrate and cadmium carbonate was observed. Result showed that reduction in oxygen consumption may be due to interaction of toxic heavy metals with respiratory system of fish by asphyxiation, abnormality in function of gills and also inhibition of enzyme system. Interactions among the metals in aquatic media are capable of producing damage in gill tissues and ultimately caused death of fish (Moolman *et al*., 2007). Any disturbance in aquatic environment could result in reduced metabolic rate and reduced growth in fish. Consequently, fish exposure to escalated concentrations of metals, resulted in high mortality rates which ultimately elevate the depletion of fish population in contaminated waters (Sarnowski, 2003).

Conclusion: The metal mixture 96-hr LC_{50} and lethal responses by both, grass carp and silver carp showed statistically significant differences. Between the two fish species silver carp was found significantly more sensitive in terms of 96-hr LC_{50} than that of grass carp. However, grass carp appeared significantly more sensitive than silver carp for lethal responses. Positively significant correlation was found among metallic ion concentrations with carbon-dioxide, sodium, potassium and electrical conductivity while inverse relationships with dissolved oxygen for both fish species.

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