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RESEARCH ARTICLE

ACUTE TOXICITY EFFECT OF COPPER AND CADMIUM IN SINGLE AND BINARY EXPOSURE ON MORTALITY RATE AND BEHAVIOURAL RESPONSES OF FRESHWATER FISH, *CYPRINUS CARPIO*

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ABSTRACT

Most of the studies reported the effects of metals on fish under the exposure to a single metal, whereas the fishes in the polluted water bodies are typically exposed to mixtures of metals. Hence, the aim of the present study was to investigate the acute toxicity of copper (Cu) and cadmium (Cd) in single and co-exposure on mortality rate and behavioural changes in freshwater fish *Cyprinus carpio*. The static and renewable bioassay method was adopted to determine the lethal concentration (LC_{50}) of tested metals through probit analysis. The 96 hr LC_{50} values were 38.36 mg/L for Cu, 92.23 mg/L for Cd and 23.90 mg/L for Cu plus Cd, respectively. The dose and time dependent increase in mortality rate was observed in *C. carpio* in response to individual and combined exposure of Cu and Cd. Behavioural response under this investigation showed prominent changes of erratic swimming activity, increase in opercular movement, exudation of mucous over the bodies, loss of equilibrium and body dispigmentation in *C. carpio* exposed to Cu, Cd and Cu plus Cd mixture at their various lethal concentrations. Comparing the 96 hr LC_{50} and behavioural changes, that Cu plus Cd was the most toxic, followed by Cu and Cd. The results further show that the single exposure of Cu was more toxic than the Cd, but in the binary exposure of Cu and Cd, both are interact with each other and enhance the toxic effect which indicates synergism. The observed data suggest that *C. carpio* considered as a good biomarker to access the metallic contaminants of freshwater aquatic bodies.

INTRODUCTION

The increasing worldwide contamination of freshwater system with thousands of industrial and natural chemical compounds, is one of the key environmental problems (Schwarzenbach *et al*., 2006). Besides numerous organic compounds entering aquatic ecosystems, heavy metal input is still rising. Cadmium is one among the heavy metals that are dispersed throughout the modern environment mainly as a result of pollution from a variety of sources (Bhattacharyya *et al*., 2000). Apart from Cadmium (Cd), Copper (Cu), a trace metal, is one of the most important pollution-causing metals. Copper and Cadmium are considered to be an important xenobiotic, persistent and non-biodegradable chemical and also known to be the most common cumulative pollutants in terrestrial and aquatic ecosystem (McGeer *et al.*, 2000). In the field, contaminants are present as mixtures instead of as an individual chemical which becomes a challenge to researchers in the field of traditional toxicology who have mainly focused on individual chemicals. The information from the studies on individual chemical on survival of fish is necessary for elucidating its mode of actions and understanding its effect caused by these chemicals.

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In aquatic toxicology the traditional LC_{50} test is often used to measure the potential risk of chemical. Although the toxicity studies and the determination of the lethal concentration for 50% (LC_{50}) of fishes have been worked out in different fish species (Roy *et al*., 2006; Ghosh *et al*., 2006; Sivakumar *et al*., 2006). The information about acute toxicity in fish abundant (Roy *et al*., 2006). The use of biological test system for monitoring pollution is gaining important worldwide (ISO, 1982) by employing toxicity test model with use of a key species of fish (Verma *et al.*, 1996). The different fish species and different type of metals were used in literature in evaluating LC_{50}, the species comparison with exposure of various metals for acute toxicity is difficult. Hence, the toxicity of metal contamination depend on type of metal, type of fish species, exposure dose, duration of exposure, etc. (Allen *et al*., 2004).

A higher concentration of Cu (or) Cd in the aquatic environment is lethal to many organisms (Eisler and Gadner, 1973; Arellano *et al*., 2000; Zyadah and Abde, 2000; Hansen *et al*., 2002; Krishnani *et al*., 2003). Bears *et al.* (2006) stated that the fish can serve as a vital indicator for metal toxicity when they continuously exposed to metals through gill respiration or ingestion of metal contaminated food. Cu and Cd toxicity in aquatic system also varies widely with water quality characteristics (Taylor *et al*., 1991; Nussey *et al.,* 1999; EPA, 2001; Straus, 2003; Rondeau *et al.,* 2005). Cu and Cd toxicity show a different profile, since increased Cd toxicity (Lydersen *et al*., 2002; Hansen *et al*., 2002) and decreased Cu toxicity (CCME, 1992; Straus, 2003) to fish have been observed in alkaline water. Copper toxicity is believed to be partly due to non-specific binding of the metal ion Cu^{2+} to biologically important molecule (Camakaris *et al*., 1999). Fish can show several behavior changes towards Cu, they may avoid it (or) orientate towards the source in a gradient of Cu (CCME, 1992). Whereas, the Cd has been shown to exert a wide range of behavioral changes in various fish species, from imbalanced swimming to decrease in the capture efficiency of prey (Yilmaz *et al*., 2004; Riddell *et al*., 2005). In contrast, interesting behavioural effects of Cd has also been reported by Sloman *et al.* (2003) with respect to dominance hierarchies in rainbow trout. The information from most of the studies on the acute lethal toxicity and behavioral response in fish used with individual metal. Polluted water bodies, however, usually contain elevated levels of various metal mixtures. The effect of mixtures of two or more chemicals may be additive, synergistic or antagonistic. Therefore, the effects of mixture of Cu and Cd on fish may also differ from their individual effects on the response of a fish. Thus, a comparison of the effects of Cu and Cd on survival response and fish behavior caused by exposure to either individual or their mixture is an interesting task. Hence, the main aims of this study are to investigate the acute toxicity range of copper and cadmium in individual and binary exposure on survival response and behavioral changes in common carp, *Cyprinus carpio* which is an edible freshwater fish of great economic importance.

MATERIALS AND METHODS

Experimental Fish

Healthy adult fish, *Cyprinus carpio* (L) with 200 ± 10 g weight and 22 ± 2 cm length were selected for the experiments. Fishes were collected from Kamaraj fish farm at Cholatharam, 30 kms away from Annamalai University. Fishes were separately maintained in laboratory at 26 ± 1^0C in a cement tank filled with dechlorinated tap water having the specific physic-chemical parameters listed in Table 1. The test water parameters were measured according to the experimental procedure described by APHA (1980). Fishes were fed with commercial fish pellet twice per day during the acclimation period. During the period of acclimation, 1/3rd water was renewed every day with filtered tap water. Feeding was suspended 24 hr before to start the experiment on mortality test for the fish.

Table 1. Physico-Chemical characteristics of tap water used for the experiments

Parameters	Unit	Value
pH	-	7.2 to 7.6
Temperature	^oC	26 ± 1
Alkalinity as $CaCO_3$	mg/L	165 - 168
Dissolved Oxygen	mg/L	7.0 to 7.5
Total hardness as $CaCO_3$	mg/L	240 to 260
Salinity	mg/L	0.3 to 0.35

Test Chemical

The test chemical used for the experiments was analar grade Copper (Cu) in the form of Copper Chloride ($CuCl_2.2H_2O$) and Cadmium (Cd) in the form of Cadmium Chloride ($CdCl_2.H_2O$), which have specification listed in Table 2. The stock solution was prepared by dissolving 3.42 g of Cu (as $CuCl_2$), 4.06 g of Cd (as $CdCl_2$) and 1.71g of Cu + 2.03g of Cd in 1 lit of deionized water to make 1000 mg /L of stock for each and then diluted with tap water to obtain the desired concentration.

Table 2. Specification of used chemicals

Specifications	Chemicals	
Chemical Compound	Copper Chloride	Cadmium Chloride
Emprical Formula	$CuCl_2.2H_2O$	$CdCl_2.H_2O$
Colour	Blue-green	White
Appearance	Solid	Crystals
Melting Point	100^{o}C	564^{o}C
Boiling Point	993^{o}C	967^{o}C
Solubility	Soluble in water	Soluble in water

Acute Mortality Test

In the present study 24, 48, 72 and 96 hr LC_{50} value and their 95% confidence limits of Cu, Cd and Cu plus Cd mixture for adult *C. carpio* were determined following probit analysis method of Finney (1971). Five to eight concentrations of each test metal (Cu, Cd and Cu plus Cd) were prepared for initial range finding tests. Definitive tests were subsequently conducted using eight to ten various concentrations for each of Cu, Cd and Cu plus Cd mixture. Each test was performed with appropriate control in duplicate using ten fish in each test tank for all the experimental groups. Feeding was suspended 24 hr before to start the experiment to avoid the possible oxidation of the toxicant used in the experiment. Fish were considered as dead when there was a lack of both opercular and body movements when probed by a glass rod (Reish and Oshida, 1986; Adeogun and Chukwuka, 2012). Dead fish were removed from the trough immediately. During this experiment the behavioral and morphological changes were also critically observed.

RESULTS

Acute toxicity test

The median lethal concentration (LC_{50}) and their confidence limits of single and binary exposure of Cu and Cd of *Cyprinus carpio* are summarized in Table 3. The LC_{50} values of copper exposure were found to be 69.42, 56.78, 42.23 and 38.36 mg/L Cu and Cd exposure were 120.41, 111.32, 104.26 and 92.23 mg/L Cd and binary exposure of copper and cadmium were 57.29, 43.72, 30.96 and 23.90 mg/L Cu plus Cd on adult fish, respectively for 24, 48, 72 and 96 hr of exposure (Figs. 1-3). Toxicity of Cu and Cd revealed that the rate of mortality was increased with the increasing in the concentration of Cu (or) Cd. The mortality data were subjected to probit analysis and plotted against the log of dose concentration resulting in a

Table 3. Median lethal concentrations (LC_{50}) and their 95% confidence limits of Cu, Cd and Cu plus Cd mixture for *Cyprinus carpio*

Metal Exposure	Exposure Time (hr)	LC_{50} (mg/L)	95 % Confidence limit (mg/L)	
			Lower	Upper
Cu	24	69.42	66.09	73.10
	48	56.78	53.50	60.18
	72	42.23	38.87	45.41
	96	38.36	35.16	41.49
Cd	24	120.41	117.24	123.96
	48	111.32	108.51	114.23
	72	104.26	101.25	107.27
	96	92.23	88.83	95.43
Cu+Cd	24	57.29	54.01	61.11
	48	43.72	40.61	47.08
	72	30.96	27.66	34.32
	96	23.90	19.99	27.60

straight line for Cu, Cd and Cu plus Cd mixture exposures (Figs. 1 to 3). Straight line is obtained at 38.36 mg/L for Cu, 92.23 mg/L for Cd and 23.90 mg/L for Cu plus Cd mixture as LC_{50} at the interval of 96 hr. The 100 per cent survival (LC_0) of fish for more than 96 hr was observed only above at a concentration of 20.50 mg/L in Cu, 45.50 mg/L in Cd and 11.50 mg/L in Cu plus Cd exposure.

Behavioural response

In the present investigation, the fish *C. carpio* exposed to lethal and sublethal concentrations of test metals of individual and binary mixture exposure, showed an abnormal pattern of behavioral response during their toxicity test.

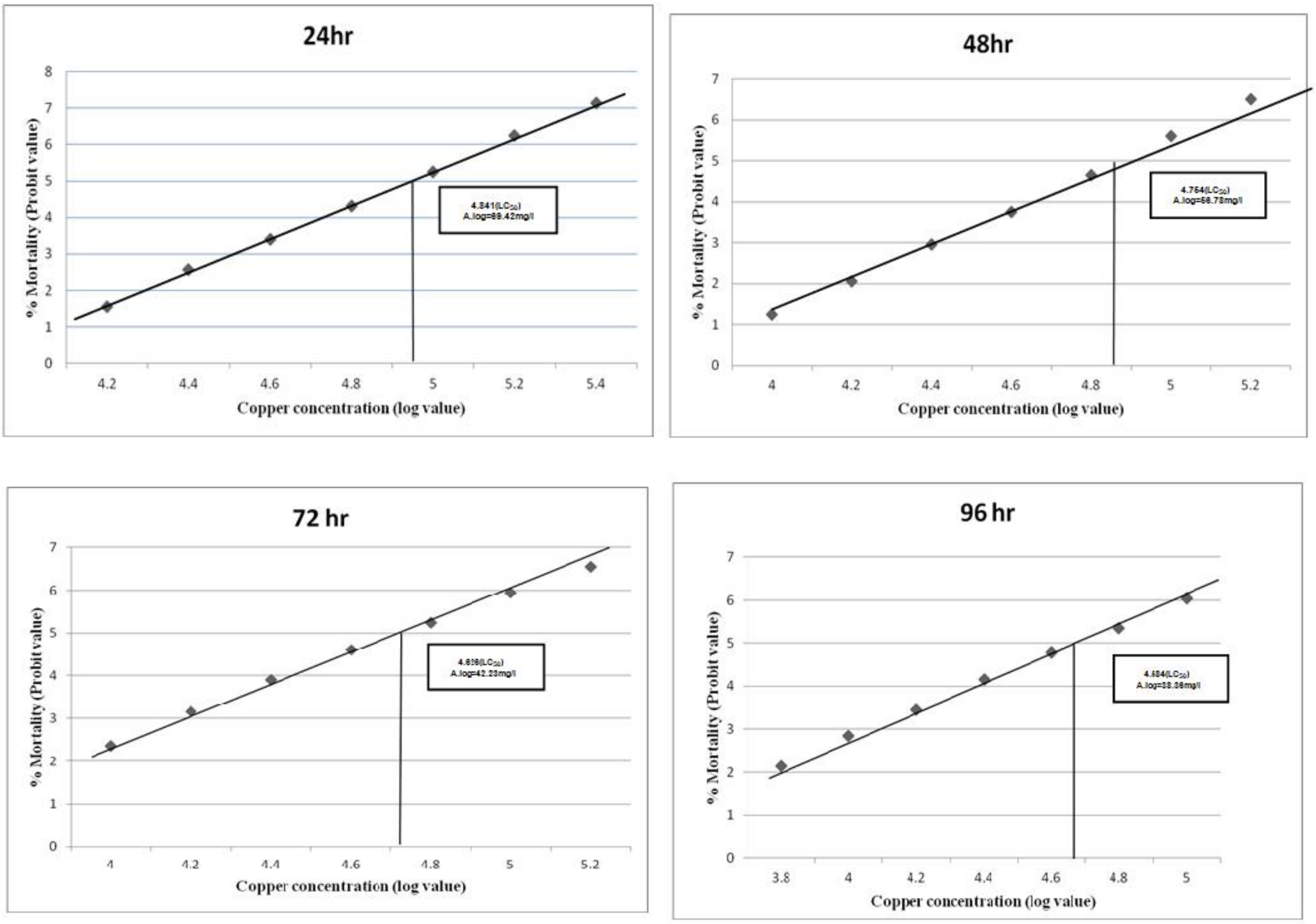

Fig. 1. Toxicity graph for the relation between concentration of copper and probit mortality of *C. carpio*

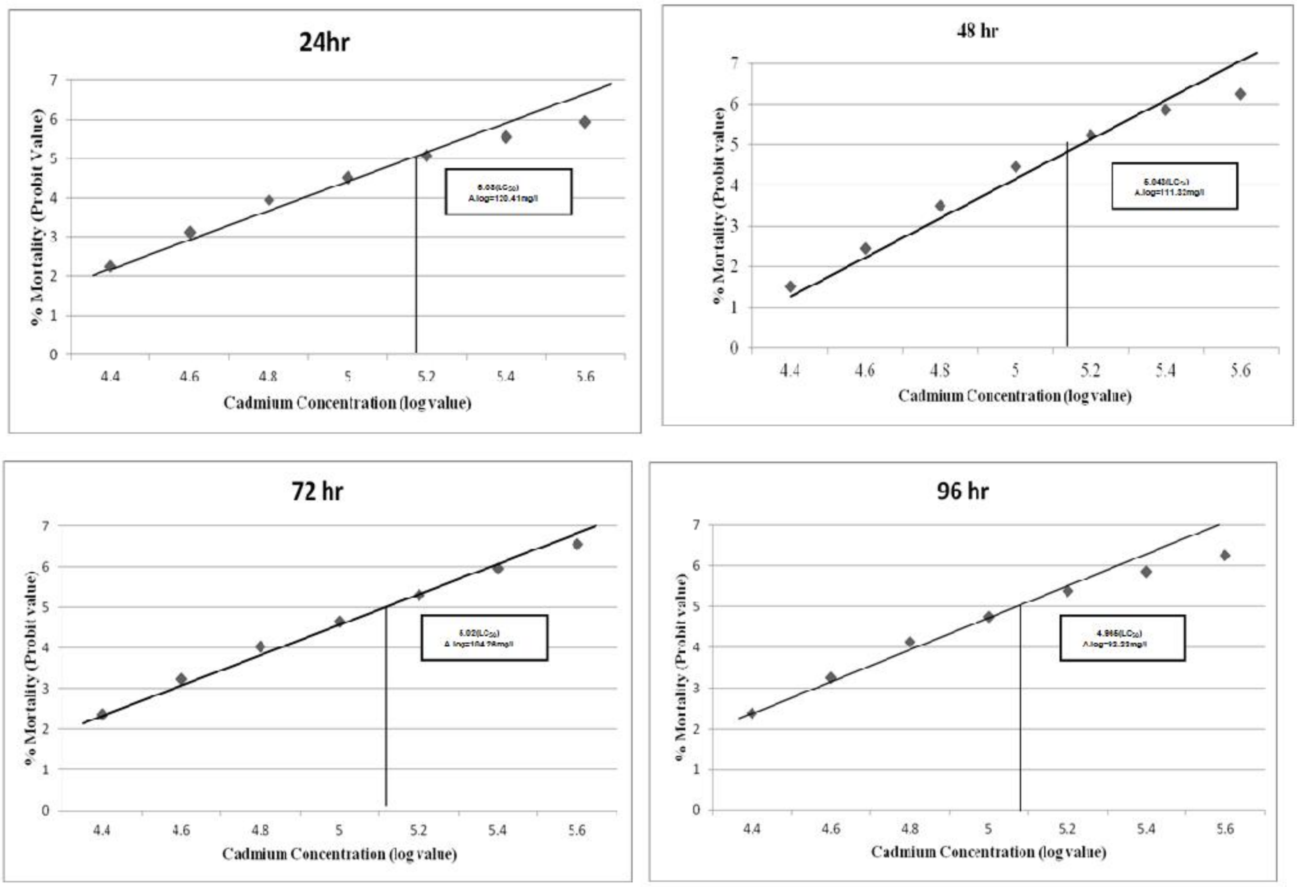

Fig. 2. Toxicity graph for the relation between concentration of cadmium and probit mortality of *C. carpio*

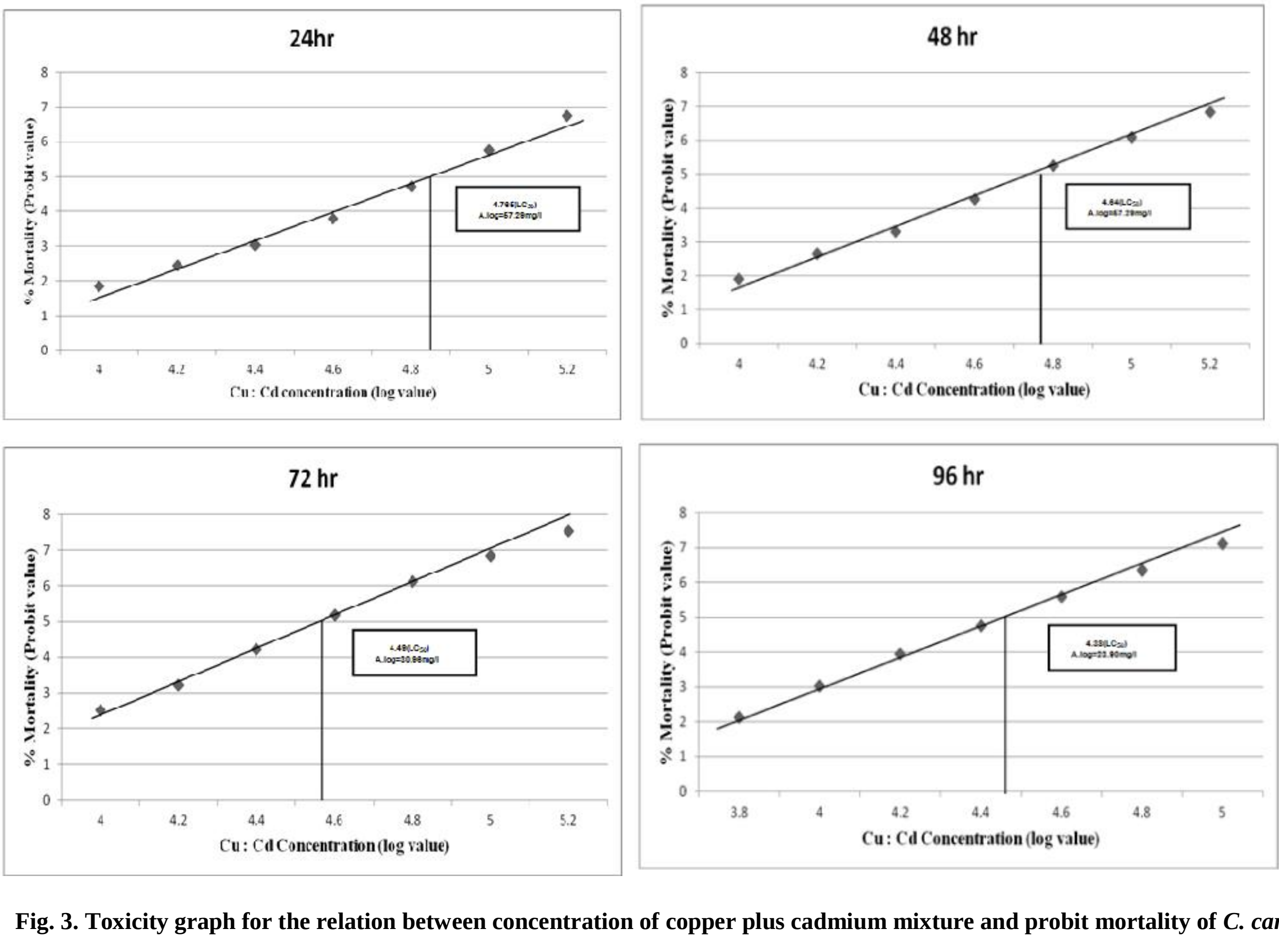

Fig. 3. Toxicity graph for the relation between concentration of copper plus cadmium mixture and probit mortality of *C. carpio*

During the initial exposure time, the fish showed some kind of hyperactive like rapid movement, faster opercular activity and often the surfacing activity at all concentrations tested with its own experimental series, possibly a form of avoidance responses. The activity was more prevalent in the binary exposure than the individual exposure of test solution. The fishes were move to near the water surface to engulp atmospheric air. Sometimes the fins became hard and also stretching of body muscle of fish in water containing test chemical. Further, the fishes in the test chemical secreted conspicuous amount of mucous from the whole body continuously during the initial period of exposure. After a few hours, body dispigmentation was also noticed in the fish in all the experimental groups. During the later periods of exposure at higher concentrations of individual and binary mixture of Cu and Cd (lethal concentration), the fishes lay on their sides or swam vertically with their tails down. This was followed by a loss of equilibrium and the fish became progressively lethargic and lost their sense of equilibrium completely under lethal level exposures. If fishes could remain in this situation for a long period, ultimately, they were laid down on the bottom of the trough with their belly upward and all of them died. No differences in behavior were noticed between the individual and binary mixture exposure. But, the activity of test fish was more frequent in the exposure of binary mixture than the individual exposure of Cu and Cd.

DISCUSSION

Acute toxicity on lethality

In the present study, acute toxicity test was used for the determination of lethal toxicity of single and co-exposure of Cu and Cd using an adult *C. carpio* as test-organism. These metals are relatively toxic even at fairly low concentration and affect the survival of fishes and other aquatic organisms (James, 1990). Mortality of aquatic animal is the most significant parameter in toxicology (Vosyliene *et al*., 2003). The toxicity test and the applications of LC_{50} values have gained acceptance among toxicologists and the toxicity test is the most highly reliable for assessing the potential adverse effect on aquatic life (Brungs and Mount, 1987). The median lethal concentration (LC_{50}) and their confidence limits of Cu, Cd and Cu plus Cd for *Cyprinus carpio* at various intervals are summarized in Table 3 and are also mentioned in Figs. 1 – 3. Results of this study revealed that the mortality rate of *C. carpio* to singly and combined form of Cu and Cd was concentration and time-dependent. Further, the mortality of fish exposed to the metal (Cu and Cd) solutions increases obviously with increasing exposure duration. The LC_{50} values and 95% confidence limits for the Cu, Cd and Cu plus Cd mixture exposures were decreased with increasing of exposure time for test fish. The current study clearly show that an increase in the concentrations of metals require the decrease of exposure time to bring about 50% mortality of fish *C. carpio*. Further the present findings revealed that the mortality of the test fish to the individual and binary exposure of Cu and Cd were dose and time dependent and it shows the regular mode of action which might be due to the magnification of these metal in tissues upto dangered level thereby lead to the death of fish. According to Karuppasamy (2004) and Mohammad *et al*. (2012) a rise in mortality rate of fish with increasing concentration reveals bioconcentration of the heavy metals under continuous exposure.

Comparison of the established 96 hr LC_{50} values for the individual exposure of Cu and Cd indicated that Cu is the more toxic than the Cd metal for *C. carpio*. It has also been demonstrated that Cu is more toxic than Cd for other species (Eisler and Gadner, 1973 and Pelgrom *et al*., 1995). James *et al.* (1990) measured the 96hr LC_{50} level of 0.67 mg L^{-1} for Cu and 5.7 mg L^{-1} for Cd to the fish *O. mossambicus* under Cu and Cd exposure. Kazlauskiene and Vosyliene (2008) also reported that in most cases Cu is the more toxic metal than Cd at all stages of development in rainbow trout (*O. mykiss*), which consistent with the present findings. The rapid action of copper in killing fish is due to its toxic effect on the respiratory function of the fish by acting as an uncoupler of oxidative phosphorylation at the mitochondrial level (Andrews *et al*., 1983). Differences in the toxicity between Cu and Cd might be due to their bioavailability that actually be absorbed by the organism (USEPA, 2001) which is in line with the results obtained here. The established 96 hr LC_{50} values among the three groups of exposure (Cu, Cd and Cu plus Cd), their toxicity on mortality response to the *C. carpio* is resulted that the joint action of Cu and Cd is the most toxic followed by Cu and the least effect of Cd. It is of interest to note that synergistic toxic effect of metal mixtures (Cu plus Cd) on adult *C. carpio* than expected on the basis of individual components. According to Stenersen, 2004, if a non-toxic single acting substance enhances the toxicity of another, it is a synergistic / additive interaction. Copper appeared to have a strong synergistic effect in combination with Cadmium and Zinc metals, e.g. LC_{20} of cadmium and copper in combination resulted in a 100% mortality of the animals in 48 hrs (Wah Chu and Chow, 2002) and a combination of zinc or cadmium with copper increases the toxic effect of copper in several times, representing a synergistic action (Metelev *et al*., 1994). Chukwu and Lawson (2009) also reported the possibility of synergistic reactions occurring when mixtures containing heavy metals interact with other pollutant mixtures present in the ecosystem. The result of the present work highly unanimous with the findings of Effendy *et al*., (2010); Obiakor *et al*. (2010) who have reported that the frequency of micronuclei in *Synodontis clarias* and *Tilapia nilotica* was heavily ($P<0.05$) observed in fish exposed to binary mixtures of the heavy metal Copper and Zinc. From the foregoing discussion it is inferred that the exposure in the metal mixture of Cu plus Cd shows the increasing of toxicity to the fish on mortality rate than their individual exposure.

However, a contrary statement has also been stated by Sarnowski and Witeska (2008) who have reported that Copper was more toxic to the fish comparing with the toxicity of either a mixture of Cu plus Cd or Cd alone. Further, they have suggested a possible antagonism of Cadmium against Copper toxicity. The above contrary statement on the joint action of Cu and Cd with the present finding is probably due to differences in profile of water used in experiments (Lydersen *et al.*, 2002 and Straus, 2003). This variation may be due to the differences in physic-chemical properties of test water and age / size of the fish. Differences in the absorption and toxicity of heavy metal could be due to age of the fish and condition of test fish along with experimental factors (Sprague, 1969). According to Subathra *et al.* (2007) that adult fish were more tolerant to copper than the juvenile fish.

Behavioral and Morphological Changes in fish

Behavioral changes have been established as sensitive indicators of chemically induced stress in aquatic organisms (Suedel *et al*., 1997; Remyla *et al*., 2008; Kaushal and Mishra, 2011). In the current study the behavior of the fishes in both the control and experimental groups were noted during the acute toxicity test. After exposure to various concentrations of the test metals (Cu and Cd), fishes showed the remarkable changes in their behavior. The toxicity intensity was more pronounced on the behavior of fish exposed to Cu plus Cd mixture than the individual exposure at all the periods of observations. The abnormal behaviours were probably caused by the neurotoxic effects and also by the irritation to receptor system of the body. On initial period of exposure at higher concentrations the fishes showed increased swimming, try to jump out from trough to avoid the metal toxicity followed by the restlessness, flickering of the eye and hyperactivity. Enzymatic as well as ionic disturbances in blood and tissue may be associated with such abnormal behavior and altered movements (Sloman *et al*., 2003). Peeling of the skin and discoloration of skin were prominent at this stage. After 48 hours the fish showed slow swimming activity in all the experimental groups, whereas the body discoloration was continuously increased. Further, a thick film of mucous on the body surface and the skin lesions around the head region, base of caudal fins and pectoral fins were prominent in 95% of the fishes after 72hr of exposure in higher concentration of Cu (or) Cu plus Cd mixture. The accumulation of mucus on the body could be connected to the intensification of mucus secretion of mucous cells activated by the toxicants (Ofojekwu, 2005; Omitoyin, 2007; Guedenon *et al*., 2012). According to Pandey *et al.* (1990) stated that the heavy exudation of mucus over the body and dispigmentation at the initial period of exposure to a toxicant is attributed to dysfunction of endocrine gland (Pituitary) under the toxic stress. Further, the accumulation and increased secretion of mucus in fish exposed Cu (or) Cd (or) Cu plus Cd may be an adaptive responses which may contribute to provide an additional protection against corrosive nature of the metal to avoid absorption of the toxicant by general body surface. This results similar to the earlier findings of Karuppasamy (2001); Sivakumar *et al.* (2006). Scale depletion, skin lesion gradually noted from dorsal to either side of the body in fish exposed to higher concentrations of metals at this stage. The injury or lesions, degenerative changes in the skin and gills of fishes under toxicant may be due to replacement of loose connective tissue elements (Banerjee, 1997). After 72 hr exposure, fish showed significantly increased surfacing activity and frequent engulfing of air from atmosphere which were more prominent under the joint action of Cu plus Cd exposure.

The fishes showed loss of balance, rolling and jerky movements during swimming under toxicant medium. Behavioral alterations like erratic swimming, restlessness and surfacing activity may be an avoiding reaction to the narcotic effects of heavy metal or to change in sensitivity of chemoreceptor's (Maruthanayagam *et al*., 2002; Atif *et al*., 2005; Kaushal and Mishra, 2011). The formation of schooling was found, it was weakening of the test animal compared to the control and fishes were slowly settled at the bottom of the trough. The decrease in opercular movement and corresponding increase in the frequency of surfacing activity of fish clearly indicates the adaptively shifts towards aerial and the fish tries to avoid contact with the metal medium through the gill chamber (Karuppasamy, 2001). Before the mortality, there was uncoordinated and vertical movement of the fish which may be due to loss of equilibrium. Uncoordinated movements shown by the fish under metal pollution could be inhibiting the action of acetyl – cholinesterase, causing death through the damage of the cerebrum and paralysis of the respiratory muscle (Modi, 1970; Hollis *et al*., 1999; Kaushal and Mishra, 2011).

Conclusion

The present observations suggest that Cu plus Cd mixture is more toxic for *Cyprinus carpio* than the individual effect of Cu and Cd which indicates a possible synergism by the interaction of Cu plus Cd mixture exposure. Further, the data in the present study substantiate the observations of James (1990) that the survival and behavioural alterations of fish, rather than the metal concentrations in the water, are suitable for environmental monitoring, expecially when trying to relate the toxic effect of metals to the biological function of fish species.

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