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Sublethal Effects of Mixtures of Copper and Ammonia on Selected Biochemical and Physiological Parameters in the Catfish *Heteropneustes fossilis* (Bloch)

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Mixtures of pollutants in the environment can influence the toxicity of each other; this influence could be different from the individual toxicological effects alone (Brown 1980; James et al. 1991; 1992). Copper salts are widely used as algicides in fisheries and fungicides in agriculture. The use of high concentrations of copper salts, however, can create pollution problems. Ammonia is the major nitrogenous end product of aquatic organisms (Colt and Tohobanoglosus 1978) and in the presence of other chemicals toxicity of ammonia is enhanced. Ammonia combines with copper to form very stable complex cations of cuprammonium $[Cu(NH_3)_4]^{2+}$ (Herbert and VanDyke 1964) and it enhances the copper toxicity. Toxic effects of individual pollutants on fish have been well documented (Sreedevi et al.1992; Mayer and Kramer 1973). However, studies on toxic effects of mixtures of copper and ammonia on fish are scanty and needs further research. In this study, individual and combined effects of ammonia and copper on selected biochemical and physiological processes in the catfish, Heteropneustes fossilis, were examined.

MATERIALS AND METHODS

Freshwater fish H.fossilis were collected from a local pond and held for 20 days in laboratory conditions (temperature: 28.5 ± 0.5°C(± SD); pH 7.8; salinity 0.15 ppt and DO 5.37 ml/l. During holding, water was changed daily and fish were fed with goat liver ad libitum. Fish (15 ± 1.5 g) were exposed to different concentrations of copper and ammonia individually and equitoxic concentrations of both; mortality was observed for 96 hr of exposure. A static renewable bioassay test was used and a 96-hr median lethal concentration was

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determined following Litchfield and Wilcoxon (1949). The 96-hr LC50 values obtained for ammonia and copper individually were 42 (95% confidence limits: lower: 33.7; upper: 65.3) and 2.4 ppm (lower:1.72; upper:3.35), respectively, and 32 ppm (lower:23.8;upper:38.4) for an equitoxic combination. Stock solutions of copper and ammonia were prepared from analar grade copper sulphate and ammonium chloride. For equal combinations of copper and ammonia, equal amounts of both stock solutions were dissolved in the medium. The concentration of ammonia in the test media was estimated following the Indo-phenol hypochlorite method (Solorzano 1969). After determination of LC50s, groups of 10 fish were exposed to 13 different exposures of copper and ammonia individually and combined (Fig.1). Duplicates were maintained for each exposure concentration and these, 20 fish were used per treatment. In the present study, 10,20, and 30% of the 96-hr LC50 values of ammonia and copper were taken as sublethal concentrations and referred to as A1,A2,A3, C1,C2 and C3, respectively. In the combi-nation study, 30% of the LC50 concentration of ammonia or copper was mixed with 10,20 or 30% of the 96-hr LC50 concentration of copper or ammonia, respectively (see Fig.1).

Fish were fed with fresh goat liver and unconsumed food was removed after 4 hr. The experiment was run for 20 days and this duration was sufficient to record signi-ficant changes in chosen parameters as revealed by pilot experiments. At the end of the exposure period, blood was collected in a watch glass containing 6% EDTA (ethylene diamine tetra acetic acid disodium salt) from 6 fish in each exposure by severance of the caudal peduncle using a sharp knife; the remaining 4 fish were used for oxygen consumption estimation. To relate blood parameters to that of biochemical parameters, chosen tissues were isolated from the same fish which were used for blood collection. Gill, liver, and muscle tissue were isolated and kept at 0°C. Similarly, blood and tissues from 6 fish at day 0 were treated as controls. The blood was used for estimating the red blood corpuscle count (RBC count) and hemoglobin (Hb) content. Hematological parameters were estimated according to routine clinical methods (Wintrobe 1978). Oxygen carrying capacity of blood was calculated by multiplying the hemoglobin content by 1.25, which is the oxygen combining power of Hb/g (Johansen 1970). The rate of oxygen consumption was estimated at 1100 hr separately in 4 fish from all exposures one day prior to the termination of the experiment following Winklers iodometric method. (APHA, 1971). The tissues of control and exposed animals were analyzed for glycogen (Kemp and Kits 1945), glucose (Roe 1955), protein (Lowry et al. 1951) and free amino acids (Moore and Stein 1954). All

the analyses were done in triplicate. Students 't' test was used to determine the significance of mean values between control and experimental groups. Correlation was applied to determine the concentration dependence of tested parameters.

RESULTS AND DISCUSSION

Exposure of H.fossilis to various sublethal levels of ammonia and copper individually and two different combinations showed concentration-dependent significant ($r = -0.993$; $p < 0.01$) reductions of glycogen content in gill, liver, and muscle, with a concomitant increase in blood glucose concentrations. The glycogen content of gills, liver and muscle of animals exposed to copper (C3) or ammonia (A3) was reduced by 23 or 17, 25 or 19, and 20 or 15% , respectively; however, it was converted into glucose and was significantly ($r = 0.994$; $p < 0.01$) elevated by 22 or 16, 26 or 20 and 20 or 14% in the same tissues, respectively, as compared to controls. Similar trends were also observed in different combinations of copper and ammonia; however, the impact was significantly ($p < 0.01$) more pronounced in copper (30 % LC50 concentration) with ascending concentrations of ammonia than ammonia (30 % LC50 concentration) with ascending concentrations of copper (Fig.2). Gill, liver and muscle showed a significant ($p<0.05$) decline of 31 or 27 % , 34 or 30 % and 26 or 22 % glycogen in C3 + A1 or A3 + C1 exposures, respectively, when compared to control animals. The reduction of glycogen reserves in tested tissues of exposed animals suggests glycogenolysis releasing glucose into the circulatory system to meet increased energy demand during stress conditions. Sastry and Gupta (1978) reported hyperglycemia in Channa punctatus exposed to mercuric chloride due to enhanced liver glycogenolysis.

Among the tested tissues,liver showed the maximum reduction of glycogen followed by gill and muscle. Physiologically, the liver requires more energy than gill and muscle for storage, interconversion, and detoxification and, hence, demands maximum energy. Next to liver, carbohydrate metabolism of the gills was most affected.

Gill, liver and muscle showed a concentration-dependent significant ($r = -0.979$; $p < 0.01$) decline in protein content and a significant ($r = 0.992$; $p<0.01$) increase in amino acid levels in H.fossilis exposed to ammonia and copper (Fig.3). This indicates proteolysis during toxic stress. The depleted proteins are utilized for various metabolic processes during stress. Kabeer (1979) reported that a decline in protein content could be due to intense proteolysis which, in turn, could

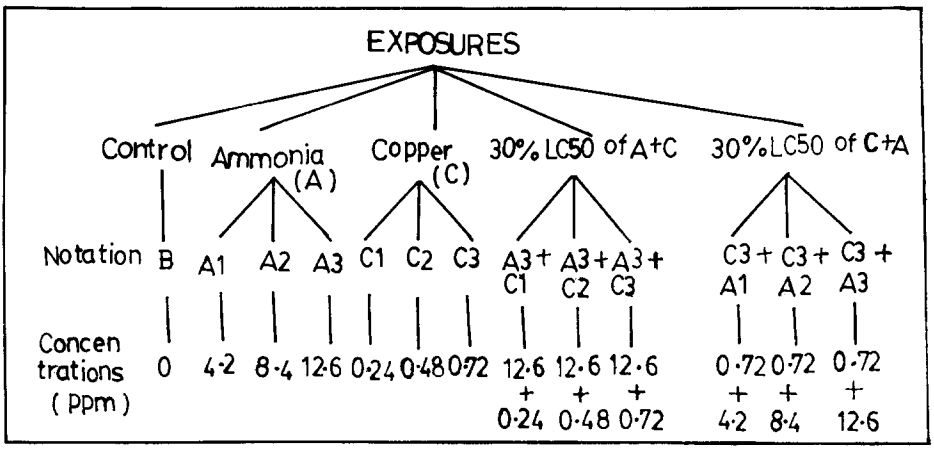

Figure 1. Experimental design indicating the 13 different copper / ammonia exposures and control.

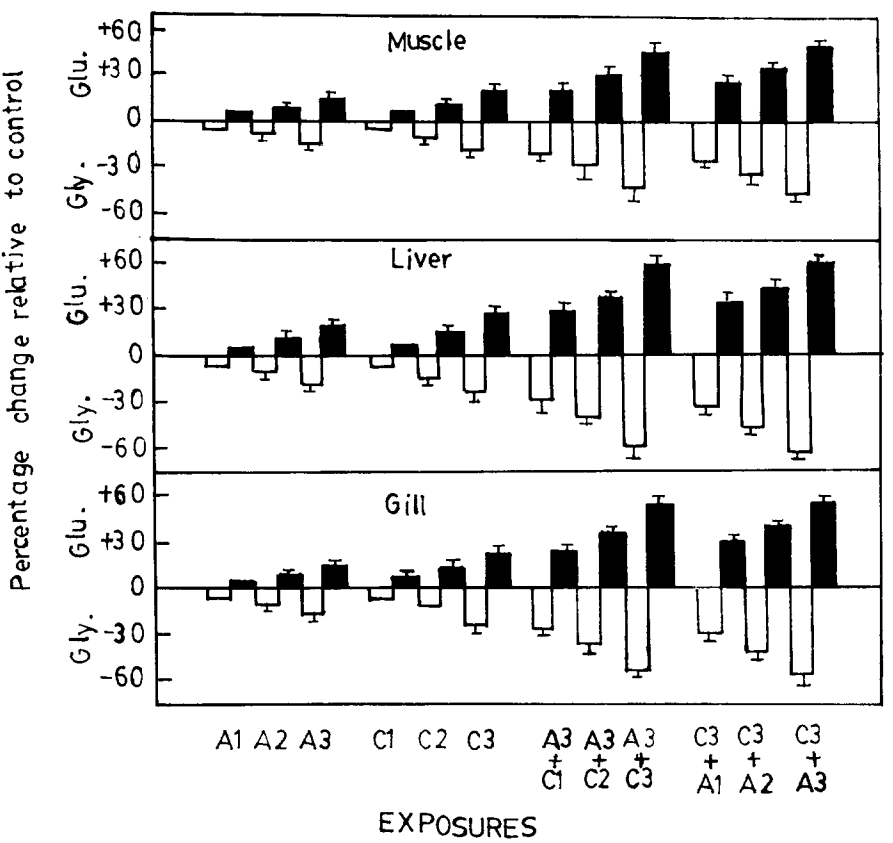

Figure 2. Effect of mixtures of ammonia and copper on glycogen (gly) and glucose (glu) changes in H. fossilis after 20 - d exposures. Each value is the mean ($\bar{x} \pm$ SD) of three estimations.

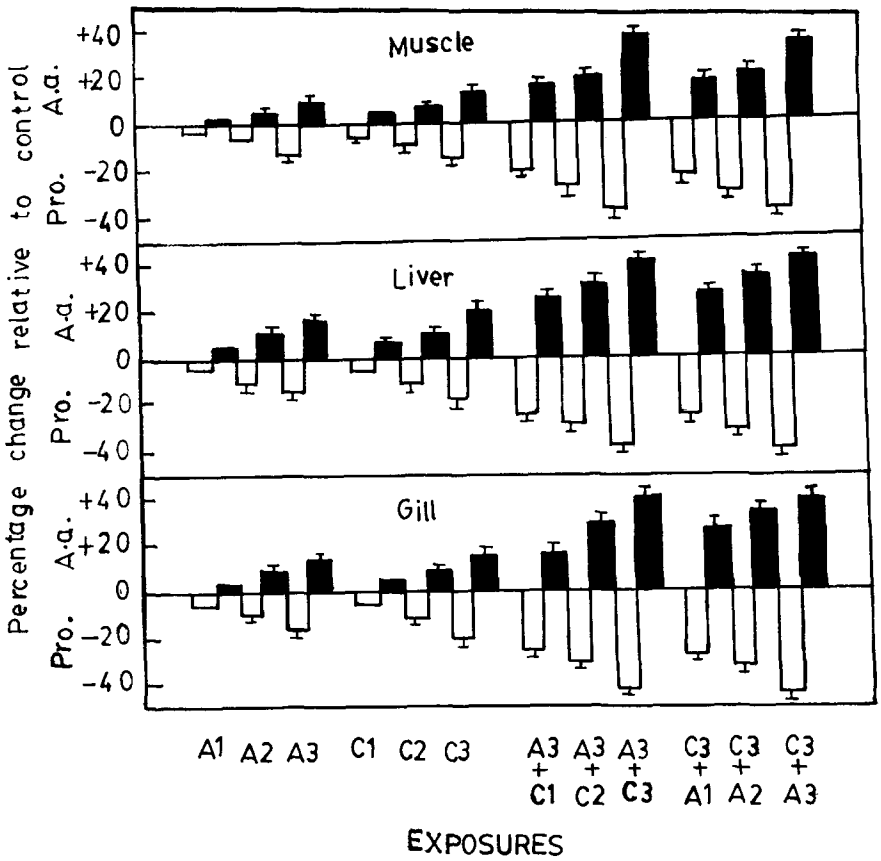

Figure 3. Effect of mixtures of ammonia and copper on protein (Pro) and amino acids (A.a.) changes in H. fossilis after 20 - d exposures. Each value is the mean ($\bar{x} \pm$ SD) of three estimations.

contribute to the increase in free amino acids to be fed into the TCA cycle as Keto acids under toxicant stress. A similar observation was also made by Sreedevi et al. (1992) in Cyprinus carpio exposed to nickel.

The RBC count of fish held in metal - and ammonia - free water was 3.18 X 10^6 / mm^3 and significantly ($p<0.05$) declined to 2.31,2.20,1.08 and 1.23 X 10^6 / mm^3 in A3, C3, C3 + A3 and A3+C3 exposed individuals, respectively (Table 1). Also, similar trends were obtained in hemoglobin content. The decrease in RBC count and hemoglobin content in H.fossilis exposed to different levels of chosen toxicants resulted in anemia. Anemia may be due to the destruction of mature RBC and/or inhibition of erythrocyte production (Wintrobe 1978) and reduction of hemo synthesis. Acute exposure of Colisa

Table 1. Effect of ammonia and copper individually and in combination on hematological parameters and oxygen consumption in H.fossilis . $\bar{x} \pm$ SD,n=3.@ $\bar{x} \pm$SD,n=4.

Exposures	RBC count ($X\ 10^6/mm^3$)	Hb content (g%)	Oxygen carrying capacity of blood ($ml O_2/gHb$)	Rate of @ oxygen consumption ($mgO_2/g/hr$)
B	3.18 ± 0.22	9.13 ± 0.12	11.41 ± 1.26	0.147 ± 0.007
A1	2.87 ± 0.31	8.03 ± 0.42	10.04 ± 0.75	0.140 ± 0.013
A2	2.67 ± 0.09	7.12 ± 0.76	8.90 ± 0.67	0.121 ± 0.005
A3	2.31 ± 0.13	6.03 ± 0.15	7.54 ± 0.82	0.107 ± 0.01
r	-0.995**	-0.999**	-0.993**	-0.986**
C1	2.97 ± 0.43	8.19 ± 0.48	10.24 ± 0.37	0.132 ± 0.012
C2	2.59 ± 0.38	7.30 ± 0.57	9.13 ± 0.50	0.110 ± 0.008
C3	2.20 ± 0.16	5.93 ± 0.41	7.41 ± 0.25	0.094 ± 0.009
r	-0.992**	-0.852*	-0.994**	-0.997**
C3+A1	2.02 ± 0.34	5.20 ± 0.10	6.50 ± 0.64	0.083 ± 0.007
C3+A2	1.58 ± 0.05	4.37 ± 0.41	5.46 ± 0.52	0.064 ± 0.007
C3+A3	1.08 ± 0.01	3.16 ± 0.04	3.95 ± 0.43	0.040 ± 0.008
r	-0.979**	-0.954**	-0.950**	-0.968**
A3+C1	1.95 ± 0.07	5.50 ± 0.03	6.88 ± 0.48	0.094 ± 0.01
A3+C2	1.62 ± 0.00	4.59 ± 0.49	5.74 ± 0.76	0.072 ± 0.001
A3+C3	1.23 ± 0.02	3.37 ± 0.06	4.21 ± 0.16	0.047 ± 0.002
r	-0.947**	-0.946**	-0.946**	-0.906**

*p<0.05; **p<0.01

fasciatus to sublethal levels of lead produced hemolytic anemia due to the lysis of erythrocytes, with concurrent decrease in hemoglobin content, hematocrit and the number of erythrocytes (Srivastava and Shashikala 1979).

Goel and Kalpana (1985) reported a significant decrease in RBC count, Hb content and hematocrit values resulting in macrocytic anemia in Heteropneustes fossilis exposed to zinc. Oxygen carrying capacity of blood also decreased in H. fossilis, and perhaps due to the significant reduction in RBC count and Hb content (Table 1). This would affect tissue respiration.

The present study revealed that the individual effect of copper was more toxic than ammonia; and two of the combinations were more toxic than the individual toxic effect of copper and ammonia. Biochemical and physiological parameters showed that when copper combines with ammonia, the toxic effect of copper was enhanced several fold. H. fossilis exposed to 12.6 ppm of ammonia (A3) significantly ($t=5.82$; $p<0.05$) reduced liver protein by 14 % and 18.1 % in 0.72 ppm of copper (C3) ($t=7.11$; $p < 0.05$) as compared to control fish. However, in fish exposed to equitoxic levels of A3 + C3, protein levels declined by 39.5 %, which is 7.4 % more than the total amount (14 + 18.1 = 32.1) of protein decline in the individual exposures of A3 and C3. This indicates the synergistic (or additive) effects of copper and ammonia (see Fig.2 & 3; Table 1). In the present investigation, all tested copper and ammonia concentrations were significant (gill glycogen: A3 + C1: $t=10.69$; $p<0.05$; C3+A1: $t=15.55$; $p<0.01$) synergistic effects. Copper has high affinity for ammonia, resulting in the formation of cuprammonium ions $[Cu(NH_3)_4]^{2+}$ and, perhaps, these could have elevated the copper toxicity. Herbert and VanDyke (1964) studied the synergistic effects of copper and ammonia and observed the formation of cuprammonium ions $[Cu(NH_3)_4]^{2+}$ of which $[Cu(NH_3)_4]^{2+}$ predominate during their interactions.

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