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Sub-Lethal Effects of Copper Sulphate on the Protein Pattern of *Clarias gariepinus* (Burchell, 1822) using SDS-PAGE

Popoola, O. M. and Ilesanmi, B. A.
Department of Fisheries and Aquaculture Technology, Federal University of Technology, Akure, Nigeria

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ABSTRACT
Copper sulphate used in agriculture as fungicide and in aquaculture for as prophylactic can subsequently cause fish mortality and also lead to the expression of stress-induced protein in fish tissue. The effects of copper sulphate were examined on 120 African catfish (*Clarias gariepinus)* juveniles with mean length and weight 71.53±5.41g and mean length 12.75±1.14 cm respectively. The test fish were acclimatized for three days in concrete tanks with well oxygenated water. Varying concentrations of copper sulphate were prepared (0.50, 0.75, 1.00, 1.25, and 1.50mg/l) and administered to the fish through immersion. After 96 hours, blood samples were collected and examined to assess the effect of exposure to copper sulphate and the level of stress posed to the fish using haematocrit, haemoglobin and blood glucose. Biochemical examination was also carried out to assess the changes in the protein pattern of fish blood using SDS-PAGE. There were significant differences ($p<0.05$) in the haematological analysis as the parameters measured differed with concentrations of the toxicant. The SDS-PAGE gel revealed wide degree of variation caused by the concentration of copper sulphate used in terms of the positions, intensity and the number of bands in the protein profiles.

Key words: protein pattern, catfish, SDS-PAGE, toxicant, stress

INTRODUCTION

Water pollution is a serious problem to all aquatic fauna and flora. In aquatic environment, pesticides may also cause several physiological and biochemical defects in fishes (Vasanthi *et al*., 1989). The aquatic environment, where fish and other aquatic organisms live, is subjected to different types of pollutants from industrial, domestic and agricultural discharge systems thereby introducing stress to the living creatures. Stress is a general and non-specific response to any factor disturbing homeostasis. Stress reaction involves various physiological changes including alteration in blood composition and immune mechanisms (Svoboda, 2001; Witeska, 2003). It has also been marked as one major factor in disease outbreaks, low productivity and mortality in aquaculture.

Stress in fish may be induced by various abiotic environmental factors (water temperature, dissolved oxygen, pH and pollution). The response to stress in fish is characterized by the stimulation of the hypothalamus which results in the activation of the neuro-endocrine system and a subsequent cascade of metabolic and physiological changes (Wedemeyer 1990; Lowe and Davison 2005). These changes enhance the tolerance of an organism to face an environmental variable or an adverse situation while maintaining a homeostasis status (Mazeaud *et al.*, 1977; Pickering, 1981).

Copper sulphate is often used as an algaecide in commercial and recreational fish ponds to control growth of phytoplankton and filamentous algae, and to control certain fish diseases (Tucker, and Robinson, 1990). However, above a specific concentration, copper is toxic to fishes, including such cultured species as Cyprinids and catfishes. Thus, treating plankton with copper compounds may lead to copper bioaccumulation reaching a toxic level in fish. The toxic effect of copper is related to its capacity for catalysing oxidative reactions, leading to the production of reactive oxygen species (Lopes *et al*., 2001).

*Corresponding Author email: omoniyipopoola@gmail.com, +2348038349325

The African catfish, *Clarias gariepinus* is a common food fish is of high commercial value in Nigeria and the West Africa sub-region. Apart from enhancing food security, its sales serve as a source of income for many families. However, the pollution of the aquatic environment can result in mass mortality of this fish even as the chronic exposure to pollutants can impair their feeding, growth and reproductive performance.

Copper sulphate is a toxic substance discharged into aquatic systems through anthropogenic activities. Consequently, there is the need to determine the degree of toxicity of the chemical to *Clarias gariepinus* juveniles. Thus, this research work was designed to evaluate the stress effects of copper sulphate on *Clarias gariepinus* juveniles. This involved determining the survival of the *Clarias gariepinus* juveniles, examining the haemoglobin, haematocrit and blood glucose levels as an indicator of stress in the blood of *Clarias gariepinus* juvenile exposed to different concentrations of copper sulphate and the effects on the protein pattern using SDS-PAGE. There is need to determine the degree of this chemicals. The objective of the study is to investigate the sub-lethal effects of Copper sulphate on protein pattern haemoglobin (Hb), haematocrit (Ht) and blood glucose of *C gariepinus* juvenile using SDS-PAGE.

MATERIALS AND METHODS

Experimental fish

One hundred and fifty healthy *Clarias gariepinus* (71.53±5.49 g weight and 12.75±1.14 cm length) juveniles were purchased from the fish farmers at Orita-obele Estate, Akure and transported live in open plastic containers to the Teaching and Research Fish Farm, Federal University of Technology, Akure, Nigeria. They were acclimatized without food for 72 hours in plastic tanks half filled with tap water prior to commencement of the experiment.

Experimental design

The stock solution of copper tetra-oxosulphate VI (Cu_2SO_4) was prepared dissolving 1g per litre of water to form 100% concentration. Five concentrations of copper sulphate (0.5, 0.75, 1.0, 1.25, and 1.5 mg/l for the treatments prepared by dilution and 0 mg/l (as control) were used for the trial. Ten juveniles of *Clarias gariepinus* were introduced into each glass tank (60 x 50 x 45 cm). The tanks were covered with mosquito nets to prevent the fish from jumping out.

The experiment lasted for 96 hours bio assay without feeding the fishes. Water quality parameters and fish behaviours were monitored while mortalities were recorded at 24, 48, 72 and 96 hours. The temperature, pH and dissolved oxygen were monitored using mercury-in-glass thermometer (Jenway 3150; dual purpose meter) for temperature (^oC), pH meter (Hanna H198106 model) and dissolved oxygen meter (Model: JPP-607), respectively.

At the end of 96 hours of exposure, fish were collected for hematological studies and blood glucose analysis. Three (3) ml blood was collected from the caudal peduncle (Stoskopf, 1993) with the aid of 20ml plastic syringe and was dispensed into a separate Ethylene Di-amine Tetra-acetic Acid (EDTA) anticoagulant bottle each for different treatments. The haemoglobin content was estimated by the acid haematin method (Sahli, 1982). The haematocript value was calculated using the micro haematocrits method described by Blaxhall and Daisley (1973). Blood glucose was estimated by a phenol sulphuric acid method. SDS-PAGE analysis was done on the blood as described by Veeraiah (2013) with little modification using the facilities of Department of Biochemistry, Federal University of Technology, Akure Nigeria. The electrophoresis gel obtained from *C. gariepinus* exposed to varying concentrations of Cu was visualized, number of bands formed, the molecular weight and intensity were recorded.

Statistical analysis

Both haematological and water quality parameters were one-way analysed of variance (ANOVA). The means obtained were separated using Duncan Multiple Range Test. analysed using Statistical Package for Social Sciences (SPSS) version 21.

RESULTS

The haematological parameters of fish exposed to different concentrations of copper sulphate after 96 hours of exposure are shown in Table 1. The mean haematocrit, haemoglobin and blood glucose of fish in the control experiment were 14.50±3.54%, 4.85±1.20 g/100ml and 0.87±0.43 mmol/L respectively. These parameters decreased progressively in the experimental fish as the concentration and the hours of exposure to CuSO4 in the water increases. The decrease was significant ($P < 0.05$) at 1.25 mg/l and 1.5 mg/l. The blood glucose was observed to increase with concentration of copper sulphate. The blood of *C. gariepinus* juvenile showed significant increase in glucose during the 96 hours bio-assay.

Table 1. Mean (± SD) haematological parameters of *Clarias gariepinus* exposed to various concentration of copper sulphate

Parameters	**Concentration (mg/l)**					
	0. 00	**0.50**	**0.75**	**1.00**	**1.25**	**1.50**
Ht (%)	14.50±3.54^{a}	10.50±7.78^{b}	7.00±1.41^{c}	6.50±4.95^{c}	5.03±5.66^{d}	5.00±0.00^{d}
Hb(g/100ml)	4.85±1.20^{a}	3.45±2.62^{b}	2.35±0.49^{c}	2.18±1.66^{d}	2.06±1.91^{d}	1.90±0.00^{e}
Bg (mmol/L)	0.87±0.43^{e}	1.56±0.63^{d}	1.75±0.11^{d}	2.78±0.15^{c}	2.92±0.43^{b}	3.17±0.39^{a}

a,b,c,d, values in each row with different superscripts are significantly different ($p < 0.05$)
Key: Ht = Haematocrit, Hb = Haemoglobin, Bg = Blood glucose

Table 2 shows the water quality parameters obtained from various concentrations of copper sulphate at 96 hours exposure of *C. gariepinus*. There was no significant difference was observed in pH and temperature among the treatments. The dissolved oxygen decreased with an increase in the concentration of the toxicant. There was a statistical difference ($p<0.05$) in conductivity.

Table 2: Mean (±SD) water quality parameters on *Clarias gariepinus* exposed to various concentrations of Copper sulphate

Concentration mg/l)	**pH**	**Temperature (ºC)**	**DO_2**	**Conductivity**
0.00	6.98± 0.06^{a}	25.43±0.99^{a}	5.53±0.35^{b}	20.39±0.52^{a}
0.50	6.95±0.03^{a}	25.40±0.95^{a}	4.80±0.77^{b}	20.69±0.43^{b}
0.75	6.95±0.04^{a}	25.39±0.93^{a}	4.75±0.77^{b}	21.35±0.85^{c}
1.00	6.96±0.04^{a}	25.52±1.04^{a}	4.33±1.03^{b}	21.09±0.33^{b}
1.25	6.94±0.04^{a}	25.42±1.00^{a}	3.99±1.20^{b}	21.47±0.24^{c}
1.00	6.93±0.04^{a}	25.47±1.01^{a}	3.43±1.38^{a}	21.65±0.23^{c}

a,b,c, values in each row with different superscripts are significantly different ($p < 0.05$) using ANOVA Post Hoc (DMRT test) (mean values, mean of fish from 3 replicate tanks).

The changes in protein banding pattern in the blood of *C. gariepinus* exposed to different concentrations of Copper Sulphate for 96 hours is shown in fig 1. The electrophotogram reveals the appearance of blood protein sub-units compared to control as the number of bands formed are more in treated fish compared to the control. Also, in comparison to control the protein subunits of Cu exposed blood revealed an increase in intensity and number as the concentration increases. These bands show distinct quantitative variation in terms of number, position and intensity of the stain.

The numbers of protein bands produced varied from six to ten and their molecular weight vary from seventeen (11kDa) to two hundred and ten kilo Dalton (210kDa). Some bands (135 and 180kDa) appeared in the treated fish compared to the control.

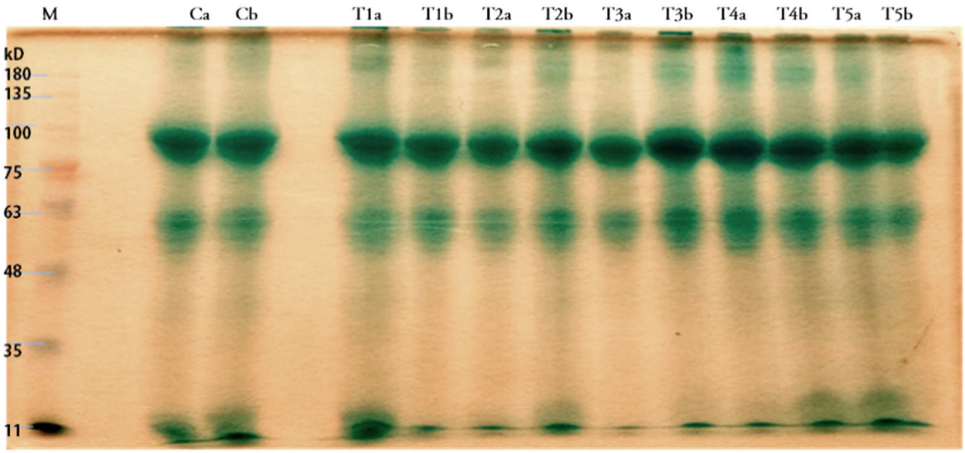

Figure 1: Changes in protein banding pattern in the blood of *C. gariepinus* exposed to sub-lethal concentrations of copper sulphate for 96 hours.

DISCUSSION

Copper is one of the heavy metals that occur naturally in aquatic environments and needed for plants and animal survival at low concentrations. The higher doses could be problematic and affect some physiological processes in living organism especially fish. Copper is an essential additive in the aquaculture business in which it is used to control algal growth as well as ecto-parasites (Tom-Petersen *et al*., 2007). The effects of Cu on living organisms are linked to total dissolved Cu concentrations (Niyogi and Wood, 2004). The bioavailability and toxicity of Cu depend on water parameters such as DO_2 concentration, alkalinity and pH and particulate organic matter (Boyd *et al*., 2005; Brooks *et al*., 2008). The result of this study shows that the increase in Cu concentration decreased the DO_2 in the aquaria.

Copper toxicity is affected by water alkalinity and hardness and when used in water containing low concentrations of $CaCO_3$, the copper ion (Cu^{++}) may cause physiological alterations in fish due to interference in the linking of ionic regulatory proteins by obstructing their regulatory functions (Adhikari, 2003). Although, copper ion (Cu++) is very essential trace metal used for metabolic functions. Figueiredo -Fernandes *et al*. (2007) has noted that it is potentially toxic when the internal available concentration surpasses the capacity of physiological detoxification processes.

Fish exposed to pollutants display a variety of physiological reactions as exemplified by blood balance disturbances (Booth *et al*., 1988). In order to understand normal and pathological processes and toxicological impacts. Sudova *et al*. (2009) affirmed that it is essential to examine the hematological characteristics in fish. Thus, the effects that CuSO4 at various concentrations posed on some of the blood indices would be understood.

Various changes in the blood indices observed in various reports could be traced to the species of fish used concentrations of toxicant and time of exposure. In this study, haemoglobin concentrations and hematocrit decreased at low concentrations (0.50 mg/l and 0.75 mg/l) but were higher at 1.25 mg/l and 1.50mg/l. Singh and Reddy (1990) has reported an increase in the haemoglobin concentration at a low Cu concentration (0.25 mg/l) in Indian catfish. Mishra and Srivastava, (1980) observed the increase in the haemoglobin concentration in fish exposed to 96 hours of 3 mg/l of Cu. In this study the increase in Cu concentration produced high levels of haemoglobin. The differences in the concentration might be difference in specie and size of the fish used.

The observed reduction in the haemoglobin and haematocrit values could be attributed to the lysing of erythrocytes. Similar observation was made by Musa and Omoregie (1999) who exposed fish to

polluted environment under laboratory conditions. However, Flos *et al*. (1987) obtained different result which could have been due to different fish species and toxicant whose reaction will differ on the fish body.

The increase in the level of blood glucose as the concentration of the toxicant increased agrees with Raja *et al*. (1992) who observed increase in blood glucose with pesticide treatment on fish due to disrupted carbohydrate metabolism as a result of enhanced breakdown of liver glycogen, probably mediated by increase in adrenocorticotrophic and glucagon hormones and/or reduced insulin activity. The increase observed might as well be due to the vulnerable stress induced by the copper sulphate resulted in hyperglycemia. Zikic *et al*. (1997) and Levesque *et al*. (2002) reported that toxicant modulate the metabolism of carbohydrates, causing hyperglycemia by stimulating the glycogenolysis in some fresh water fish species

Stress is an energy requiring process and the animal assembles energy substrates to survive stress metabolically (Vijayan *et al*. 1997). Glucose, being one of the most sensitive indices of the stress state of an organism, its high levels in blood indicate that the fish is in stress and it is intensively using energy reserves; glycogen in liver and muscles (Vosyliene, 1999). Monteiro *et al*. (2005) also, reported that plasma glucose level as stress indicator increase with waterborne Cu exposure.

The variations in protein subunit band patterns may be due to change in the turn over synthesis degradation of various proteins. This finding contradicts the result of Marinovich *et al*. (1994) that Diazinon induced inhibition of proteins in HL 60 cells at 24 hours exposure. Sherif *et al*. (2009 also observed slight decrease in intensity of proteins in Diazinon treated Nile Tilapia. Jyothirmayee *et al*. (2006) noticed a contrary result in *Clarias batrachus* exposed endosulfan. The explanation to this is that these proteins were highly affected by the stress caused by the pesticides. In this study, the observed increase in the bands and intensity of protein could be traced to the fish species and size and toxicant used. The Cu might have helped the expression of some genes and probably caused the others to yield specific mRNAs which may subsequently be translated into specific proteins called stress-induced proteins as observed in Cu treated fish (Daniel *et al*., 2004; Ksenia *et al*., 2008; Murat *et al*., 2009).

The changes in protein metabolism have been reported in fish exposed to various types of environmental stressor like metals and pesticides (Alexssandro *et al*. (2009); Shweta and Gopal, 2009). Tripathi and Shukla (1990) have reported the appearance of new proteins after exposure of the fish to pesticides to be a clear indication of the alterations in the cytoplasmic proteins.

Conclusion

The present results indicate that a short-term exposure to different Cu concentrations induced stress reaction in fish. The changes observed showed that the stress reduced the immune potential of fish. This reduced immunological status would have resulted in mortality, especially at higher concentrations. Hence, it seems that even an incidental toxic stress may cause a significant increase in susceptibility of fish to infections. Therefore, good knowledge of fish response to various stressors will be of greater help in improving the production of fish and in providing information on ways through which stress in aquaculture can be effectively controlled and monitored. The changes in the haematological parameters indicate that they can be used as indicators of Cu related stress in fish on exposure to elevated levels in the water.

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