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Characterization of protein profile in the Asian sea bass, *Lates calcarifer* (Bloch) exposed to copper

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Abstract
Characterization of protein in the metabolically active tissues of muscle, gills and liver of the Asian sea bass, *L. calcarifer* on exposure to two sub-lethal doses (6.83 and 13.66 ppm) of copper were studied for 28 days of exposure (DoE). The electrophoretic pattern of muscle and gill proteins revealed 10 and 6 slow moving bands (control). The number of protein bands decreased to 7 and 6 in muscle, 5 and 4 in gills after 7 days of exposure to lower and higher concentrations of copper, respectively. After 28 days, the protein bands decreased to 6 and 5, 1 and 1in muscle and gill tissues at different concentrations of copper. In liver tissue protein concentration is very low so could not revealed any bands.

Keywords: Copper, *Lates calcarifer*, Protein profile.

1. Introduction
The electrophoretic techniques are promising tools for identifying protein profile in response to stress and sublethal level of heavy metals. Heavy metal binding proteins have been found to be associated with copper and the lower molecular weight protein in the lake fauna [1]. Metal-binding proteins such as ferritin, ceruloplasmin, and metallothioneins (MTs) have special functions in the detoxification of toxic metals, and also play a role in the metabolism and homeostasis of essential metals [2]. Metallothioneins are low molecular weight proteins rich in cysteine residues that can bind various metals, including mercury, silver, copper, cadmium, lead, zinc, and cobalt, with varying affinities [3]. It has been reported that different fish species possess different isoforms of MTs [4]. Metallothioneins are involved in the regulation of the essential metals like copper and zinc and in the detoxification of non-essential metals [5]. Zinc has an essential function in the activation of metal-regulated transcription factors which initiate expression of the MT genes [6].
Copper plays an essential function in a variety of metabolic processes. It is a component of many enzymatic and structural proteins, including Cu-Zn SOD, cytochrome oxidase, and ceruloplasmin. Copper occurs naturally in soil and water. Mining, industrial discharges, and copper-based pesticides, especially algaecides, are sources of water contamination [7]. Copper toxicity to fish and its bioavailability in water vary with physicochemical properties of water, i.e., pH, alkalinity, suspended solids, organic compound content, and hardness [8]. The concentration of free copper, cupric ion (II) increases with water acidity. Copper hydroxide predominates in water of pH 8.0 and higher [9]. Calcium, as a contributor of water hardness, was shown to reduce the harmful effects of copper on the growth of Nile tilapia [10].
Copper plays a protective role against oxidative damage caused by variety of xenobiotics. The antioxidant effects of ceruloplasmin and metallothioneins seems to be the mechanism by which copper protects under these conditions [11]. Ceruloplasmin serves as a transport protein of copper in plasma. Parvez *et al.* [12] reported that copper pre-exposure increases the activity of ceruloplasmin in fish serum. Ceruloplasmin, through ferroxidase activity, is involved in iron homeostasis and acts as an antioxidant in plasma [13, 14]. Copper is able to induce the biosynthesis of metallothioneins [6]. Ahmad *et al.* [15] reported that metallothionein induction plays a role in the oxidative defence against chronic copper exposure in the liver of a freshwater catfish *Channa punctatus*.
The components of antioxidant defences are diversely influenced by metals. Both increase and decreases in enzyme activities and also enhanced and reduced levels of non-enzymatic

components have been described after metal exposure. A specific biomarker of oxidative stress caused by metals does not exist, and for that reason a complex approach should be taken. Metallothioneins seem to be a suitable biomarker of metal exposure, especially under laboratory conditions. In field studies the applicability of MT content in fish tissues as a biomarker is questionable following chronic metal exposure. In several field studies there were no significant correlations found between MT content and cadmium as well as between MT content and mercury in fish tissue [16]. Lobster metallothioneins share a number of similarities with mammalian metallothioneins with respect to the presence of copper and cadmium, apparent molecular weights and amino acids composition, but differ substantially in their electrophoretic behaviour [17]. Krishnamoorthy and Subramanian [18] reported intensities of major polypeptide bands in the freshwater prawn, *Macrobrachium lamarrei lamarrei*, exposed to copper. Similarly, the reduction in the number of protein fraction in *Scylla serrata* treated with copper was reported by [19]. Fish can be used as bioindicators of metals in the environment by studying the induction of oxidative stress; however, the specific forms of biomarkers and mechanisms of their action still need to be investigated.

2. Materials and Methods

2.1 Experimental fish

Healthy hatchery reared three months old juvenile Asian sea bass, *L. calcarifer* with mean total length of 7.06 ± 0.15cm and mean total weight of 10.18 ± 0.24gm were obtained from the Rajiv Gandhi Centre for Aquaculture, Thirumullaivasal near Sirkali, Nagapattinam Dist, Tamil Nadu, India. Fishes were acclimatized for 2 weeks in stock tank to the experimental glass aquaria (120x50x50 cm) filled with 250 l of water with a salinity of 27±2 ppt, under a natural photoperiod 12 h:12 h (light: dark) cycle. The water in the tanks was passed through a 1µm filter, UV-sterilized, and refilled daily. Fishes were fed twice daily with commercially prepared sea bass pellet feed which contains 2.5mg/kg of copper. They were starved for 24 h before and during the experiment.

2.2 Chemical

For preparing stock solution 3.9 gram of Copper ll sulphate pentahydrate ($CuSo_4$ 5 H_2O) (Merck) was dissolved in one litre of double distilled water and used as the stock solution. It was stored in a clean standard flask at room temperature, in the laboratory.

2.3 Experimental Procedures

2.3.1 Test Concentration

Fish were exposed to nominal 6.83ppm and 13.66ppm as copper. The doses were theoretically sublethal, 10% and 20% respectively, of the Maximum Acceptable Toxicant Concentration (MATC), which was 68.3ppm. The MATC was represented as NOEC (No-Observed Effect Concentration) <MATC <LOEC (Lowest Observed - Effect Concentration). The test concentration was estimated using the Application Factor (AF) concept, by dividing the limits (NOEC and LOEC) of the MATC by the 96-h LC_{50} (AF=MATC/ LC_{50}= (NOEC-LOEC)/LC_{50}).

2.3.2 System design

The recirculation closed system was set up according to Muthuwan [20]. The experiment was carried out in 360 l glass aquarium (120x60x50 cm), in which one compartment (50x50x40 cm) was partitioned by plastic gauze (mesh size 1.5 mm) to contain biofilter. Each aquarium was filled with 300 l of natural sea water (salinity of 27±2 ppt), which was pumped continuously over a biofilter column at the rate of 4 l/min. The water was continuously aerated throughout the experiment.

2.3.3 Test Procedure

After 2 weeks of acclimatization in a holding tank, ten healthy fish (8.06 ± 0.19cm in length and 11.18 ± 0.67gm in weight) were transferred to each aquarium at a loading density of 0.69 g/l. Three replicates were performed for test concentration and control. Fish were fed twice daily with chopped fresh fish at 10:00 and 14:00. Uneaten food was quickly removed from the system. Fish were starved for 24 h before sampling. The experimental water (50%) was changed every 2 weeks to keep the water quality in acceptable limits according to APHA [21], Water quality (dissolved oxygen, temperature, pH and salinity) was measured daily and water chemistry (ammonia nitrogen, nitrite nitrogen, nitrate nitrogen) was measured twice weekly using the Merck water quality analyzer kit. The ammonia nitrogen and nitrite nitrogen levels were controlled not to exceed 0.2 mg/l for exchanging the water in 25 percentages. The actual concentration of copper was measured weekly before and after its addition to maintain the copper concentrations at the designed level. Mortality and behaviour were observed daily in each concentration. Two fishes from each aquarium were sampled at 0, 7, and 28 days post-exposure.

2.3.4 Characterization of Protein profile

The Asian sea bass were exposed to 6.83ppm and 13.66 ppm concentrations of copper for 28 days. After 0, 7 and 28 days, the Asian sea bass were sacrificed. Muscle, gills and Liver were excised out and analyzed for characterization of protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the protocol [22]. The gel was immersed in 5 ml of staining solution (200 mg Coomassie brilliant blue R250+50 ml MeOH (Methyl hydroxide) + 7 ml acetic acid + 43 ml distilled water and was allowed to stain for 4 hours at room temperature. The stain was removed and the gel de-stained with acetic acid and methyl hydroxide solution (7 ml acetic acid + 30 ml Methyl hydroxide + 63 ml distilled water). The gel was stored in 7% acetic acid; the bands were visualized under UV trans-illuminator.

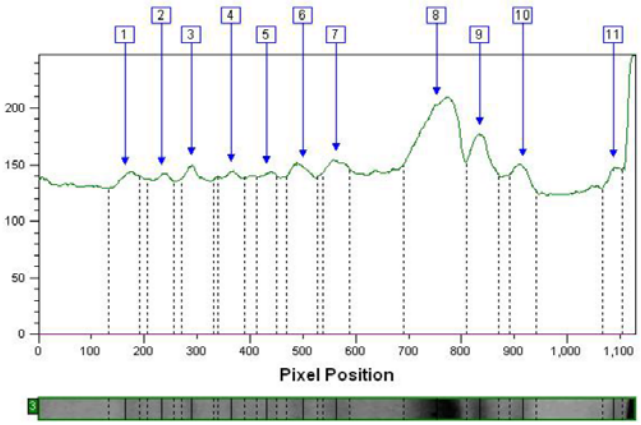
3. Results

SDS-Poly Acrylamide Gel Electrophoresis was used to find out the effects of the copper treatment on protein profiles of muscle, gill and liver tissues of the fish, Asian sea bass *(L. calcarifer*) using standard marker protein. The changes in the protein profile due to the treatment, is presented in the figures 1 to 10. In the SDS-PAGE separation, the muscle and gill tissues alone showed clear polypeptide bands. The overall results clearly indicated that certain proteins slightly increased in quantity when the treatment time was increased. However an increase in concentration resulted in a slight decrease in the quantity of different polypeptides in each tissue sample. The densitometry analysis data also presented in figures 1 to 10.

3.1 Protein Profile in muscle tissue

In muscle tissue of control group, eleven polypeptide bands were detected. The molecular weights of these eleven polypeptides were: 183.401, 162.873, 146.280, 127.263,

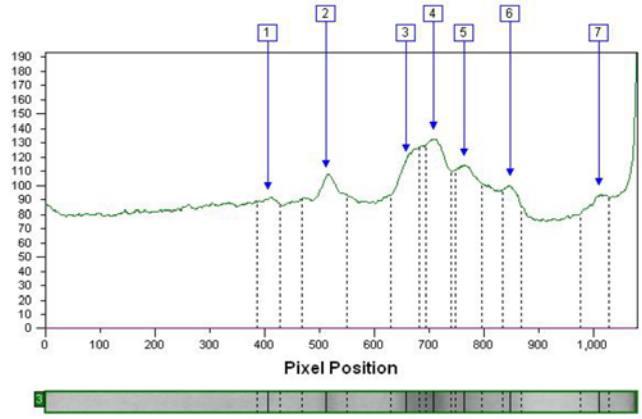
113.377, 102.290, 94.038, 67.306, 49.977 and 32.571 KDa (Fig.1). In 7 day-old treated fishes, the muscle tissue were different in total number of polypeptide bands. At 6.83ppm concentration of copper, seven bands (121.747, 88.485, 56.260, 50.906, 47.090, 40.617 and 24.0 KDa) (Fig. 2) were detected by the densitometry analysis, where as in 13.66ppm concentration treated muscles, six bands (220.508, 199.701, 178.702, 145.534, 86.915, 68.091 KDa) were detected (Fig. 3). The muscle tissue from 28 days old treated fishes also showed variation in total number of polypeptide bands due to two different concentrations. At 6.83ppm concentration, the tissue showed the presence of six polypeptides (179.668, 145.289, 93.649, 73.629, 67.994 and 61.251 KDa) (Fig. 4). At 13.66ppm concentration the numbers of polypeptide bands in muscle tissue were reduced to five with molecular weights of 186.820, 152.039, 94.597, 74.713 and 69.195 KDa (Fig. 5).



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	MW	Rf
1	617,519.00	617,519.00	-	183.401	0.146
2	536,981.00	536,981.00	-	162.873	0.206
3	642,674.00	642,674.00	-	146.280	0.257
4	534,050.00	534,050.00	-	127.263	0.324
5	408,636.00	408,636.00	-	113.377	0.383
6	662,039.00	662,039.00	-	102.290	0.445
7	582,982.00	582,982.00	-	94.038	0.500
8	1,676,248.00	1,676,248.00	-	67.306	0.667
9	742,984.00	742,984.00	-	49.977	0.741
10	542,942.00	542,942.00	-	32.571	0.812
11	397,609.00	397,609.00	-	0.000	0.965

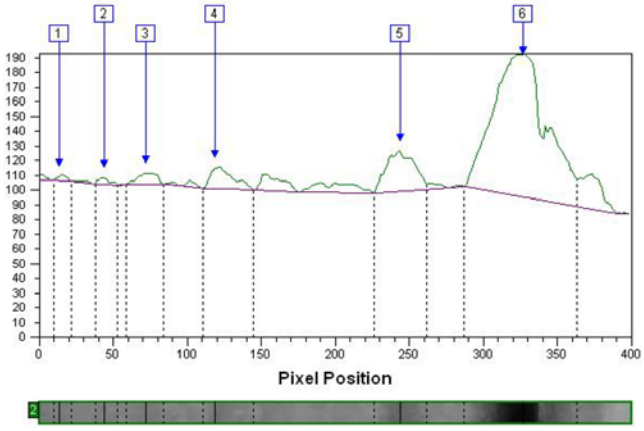
Fig 1: Electropherogram and densitometric scan images of protein profile in the muscle tissue of *L. calcarifer* (Control).



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	MW	Rf
1	225,646.00	225,646.00	-	121.747	0.377
2	468,890.00	468,890.00	-	88.485	0.475
3	353,092.00	353,092.00	-	56.260	0.610
4	338,872.00	338,872.00	-	50.906	0.657
5	315,802.00	315,802.00	-	47.090	0.709
6	196,144.00	196,144.00	-	40.617	0.786
7	280,690.00	280,690.00	-	24.000	0.937

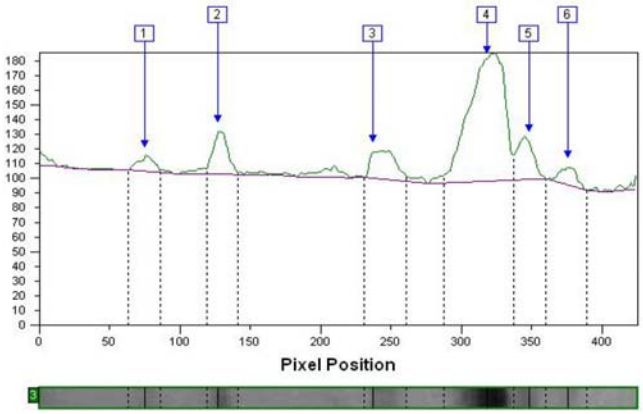
Fig 2: Electropherogram and densitometric scan images of protein profile in the muscle tissue of *L. calcarifer* after 7 days of exposure to 6.83ppm concentration of copper.



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	MW	Rf
1	521.00	26,063.00	-	220.508	0.035
2	776.25	31,935.00	-	199.701	0.110
3	2,304.50	54,317.00	-	178.702	0.180
4	5,127.00	73,688.00	-	145.534	0.298
5	12,120.20	83,528.00	-	86.915	0.612
6	81,603.65	227,349.00	-	68.091	0.820

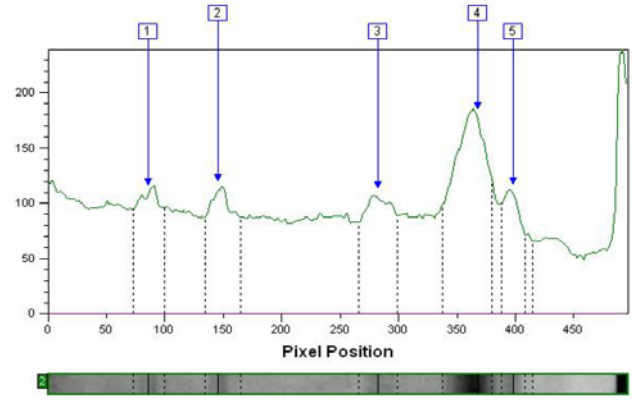
Fig 3: Electropherogram and densitometric scan images of protein Profile in the muscle tissue of *L. calcarifer* after 7 days of exposure to 13.66 ppm concentration of copper.



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	MW	Rf
1	3,227.67	61,111.00	-	179.668	0.177
2	8,684.63	62,901.00	-	145.289	0.300
3	9,512.67	81,025.00	-	93.649	0.559
4	61,186.99	176,177.00	-	73.629	0.750
5	9,688.02	64,461.00	-	67.994	0.821
6	4,111.00	70,890.00	-	61.251	0.887

Fig 4: Electropherogram and densitometric scan images of protein profile in the muscle tissue of *L. calcarifer* after 28 days of exposure to 6.83ppm concentration of copper.



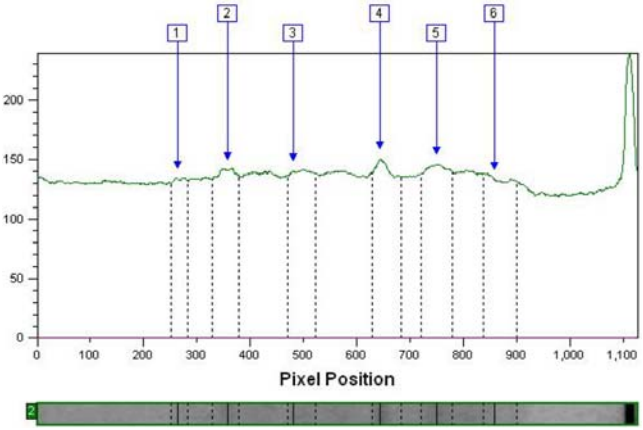
Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	MW	Rf
1	78,580.00	78,580.00	-	186.820	0.174
2	84,159.00	84,159.00	-	152.039	0.295
3	90,824.00	90,824.00	-	94.597	0.570
4	175,671.00	175,671.00	-	74.713	0.743
5	56,086.00	56,086.00	-	69.195	0.804

Fig 5: Electropherogram and densitometric scan images of protein profile in the muscle tissue of *L. calcarifer* after 28 days of exposure to 13.66 ppm concentration copper.

3.2 Protein Profile in gill tissue

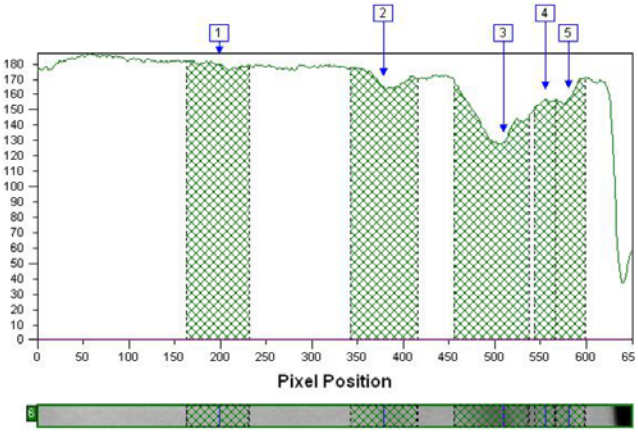
In gill tissue of control group, six polypeptide bands were detected. The molecular weights of these six polypeptides were: of 153.409, 128.711, 105.195, 83.707, 67.306 and 44.143 KDa (Fig. 6). In 7 day-old treated fishes, the gill tissues were different in total number of polypeptide bands. At 6.83ppm concentration of copper, five bands (82.512, 40.210, 22.345, 19.242 and17.572 KDa) (Fig. 7) were detected by the densitometry analysis, where as in 13.66ppm concentration treated gills, four bands (82.868, 51.574, 46.179 and 40.255 KDa) were detected (Fig. 8). The gill tissues from 28 days old treated fishes also showed variation in total number of polypeptide bands due to two different concentrations. At 6.83ppm concentration, the tissue showed one polypeptide band (15.388 KDa) (Fig. 9). At 13.66ppm concentration the gill tissue showed one polypeptide band with the molecular weight of 15.326 KDa (Fig.10).



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	MW	Rf
1	272,590.00	272,590.00	-	153.409	0.235
2	453,936.00	453,936.00	-	128.711	0.318
3	473,351.00	473,351.00	-	105.195	0.427
4	489,454.00	489,454.00	-	83.707	0.572
5	539,158.00	539,158.00	-	67.306	0.667
6	520,759.00	520,759.00	-	44.143	0.764

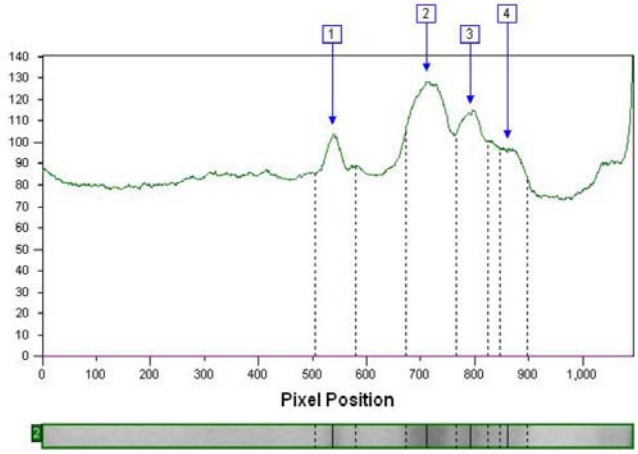
Fig 6: Electropherogram and densitometric scan images of protein profile in the gill tissue of *L. calcarifer* (Control).



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	Norm'd Vol	MW (kd)	Rf
1	817,071.00	817,071.00	-	0.00	82.512	0.307
2	833,113.00	833,113.00	-	0.00	40.210	0.584
3	763,420.00	763,420.00	-	0.00	22.345	0.786
4	225,016.00	225,016.00	-	0.00	19.242	0.855
5	350,254.00	350,254.00	-	0.00	17.572	0.895

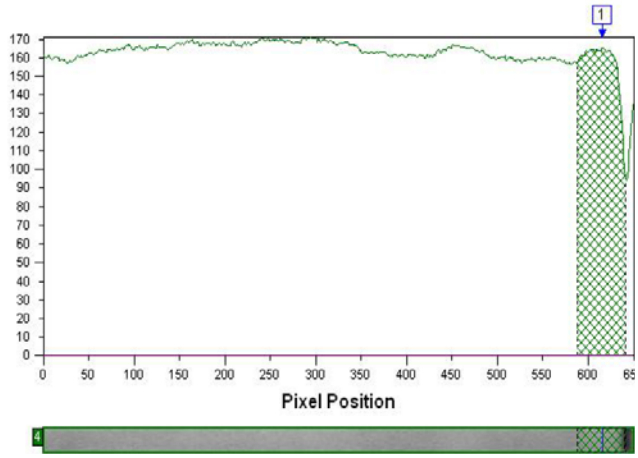
Fig 7. Electropherogram and densitometric scan images of protein profile in the gill tissue of *L. calcarifer* after 7 days of exposure to 6.83 ppm concentration of copper.



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	MW	Rf
1	433,822.00	433,822.00	-	82.868	0.493
2	697,077.00	697,077.00	-	51.574	0.651
3	404,303.00	404,303.00	-	46.179	0.725
4	300,604.00	300,604.00	-	40.255	0.789

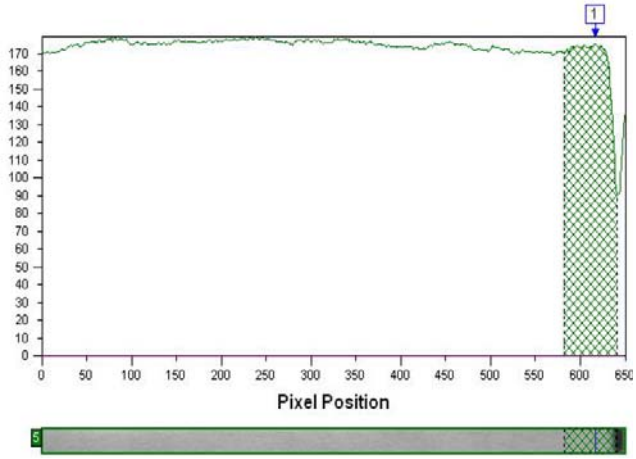
Fig 8: Electropherogram and densitometric scan images of protein profile in the gill tissue of *L. calcarifer* after 7 days of exposure to 13.66 ppm concentration of copper.



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	Norm'd Vol	MW (kd)	Rf
1	548,892.00	548,892.00	-	0.00	15.388	0.949

Fig 9: Electropherogram and densitometric scan images of protein profile in the gill tissue of *L. calcarifer* after 28 days of exposure to 6.83 ppm concentration of copper.



Band Table

Band No	Volume	Vol+BkGnd	Calib Vol(ug)	Norm'd Vol	MW (kd)	Rf
1	646,947.00	646,947.00	-	0.00	15.326	0.951

Fig 10: Electropherogram and densitometric scan images of protein profile in the gill tissue of *L. calcarifer* after 28 days of exposure to 13.66 ppm concentration of copper.

3.3 Protein Profile in liver tissue

In liver tissues, the protein concentrations were very low and separation of polypeptides was also very poor. Hence the densitometer could not detect any bands in liver tissues in control and treated groups.

4. Discussion

Heavy metal binding proteins are associated with copper and the lower molecular weight protein in particular is found to have a significant percentage of copper contained in the muscle and gills. The lower molecular weight protein probably plays a significant role in the copper metabolism. In the present study SDS polyacrylamide gel electrophoresis was performed for the tissues of muscle and gills of *L. calcarifer* exposed to sub-lethal concentrations of copper. When compared to control the protein subunits of muscle and gills exposed to sub-lethal concentrations, the bands showed decrease in intensity. The changes in protein subunit band patterns may be due to change in the turn over (Synthesis /degradation) of various proteins. The disappearance of bands in the muscle and gills of *L. calcarifer* on exposure to copper may be due to the interference of copper in the protein synthesis process as reported in freshwater prawn, *Macrobrachium lamerrei lamerrei* [18]. In the present study eleven distinct bands are accounted in the muscle tissue of *L. calcarifer*. Extensive disruption in the number of banding is well documented at 13.66ppm of concentration with six and five polypeptide fractions during 7 and 28 days of copper exposed sea bass. Similar reduction in the number of banding pattern of muscle tissue is reported in *M. lamerrei lamerrei* [18]. He also reported that the intensities of the major polypeptide bands in the gills of prawns when treated with heavy metal were less than that of the control. The result is in accordance with the current observation in *L. calcarifer* in which the six distinct bands (control) decreased to five and one at 6.83ppm during 7 and 28 days respectively. Similarly, the bands reduced to only one at 28 days of exposure to 13.66ppm concentration of copper. Similar reduction in the number of protein fractions was found in *Scylla serrata* and *Panulirus homarus homarus* when treated with copper [19, 23]. *L. calcarifer* has minimum protein residue in muscle and gills due to copper toxicity which interfered with the banding pattern of proteins as reported in other aquatic invertebrates [24]. It is also evident that exposure to copper disturbed the banding pattern of protein under stress condition in *L. calcarifer*.

The inhibition or activation of physiological activities by copper is due to the interaction between the animal and the heavy metal. The stress induced biochemical changes can be described as secondary responses of the fish. Sharaf-Eldeen and Abdel-Hamid [25] found that the exposure of *O. niloticits* to the pollutants ($CuSo_4$, Malathion and Paraquat) induced disappearance of certain serum protein fractions. Anees [26] found that the total serum protein of *Channa punctatus* decreased significantly on exposing to some organophosphorus compounds. Patterson [27] mentioned that the pollutants react with the cell nucleoproteins and nucleic acids and consequently affect the protein synthesis and cellular integrity. However, effects of toxicants on energy conservation by mammalian mitochondria have never been reported. In several toxicological models, movement disorders were linked to a dysfunction of the mitochondria.

Kurbanova *et al*. [28] reported that a decrease of the intensity of total protein accumulation and albumin concentration, and the increase of gamma globulin and peptidase activity which considered as adaptive reactions of the fish, *Rutilus Jrisii kutum* to the oil pollution. Khalid M. Sharaf-Eldeen *et al.* [29] reported that fractions of liver proteins were changed in the fish, *Tilapia zillii* when exposed to agricultural and industrial drainage water. Tripathi and Shukla [30] observed alterations in the cytoplasmic protein pattern of fish *Clarias batrachus* by performing electrophoresis of the cytoplasmic protein fractions of the liver and the skeletal muscle exposed to endosulfan and methyl parathion for 28 days. These authors also found that the disappearance and polymorphism of protein fractions were dependent on the degree of pollution in each water locality. Protein heterogeneity is associated with all fish species. Structurally blood serum protein, muscle protein (myogen) haemoglobin, as well as all enzymes in the blood and other organs of fishes appear to be variable [31]. Sanders [32] detected inter- and intraspecific differences in protein compounds. Changes in protein sub units are regarded as important biomarkers of the metabolic potential of cells, as these play the main role in regulating the activities of cells. Their ratios also provide significant information about the way in which, mechanism, these contents regulate the multifaceted activities of cells.

5. Acknowledgement

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