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Evaluation of biochemical changes and estimation of protein quantity following the treatment of cadmium in a fresh water cat fish, *Clarias batrachus*

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Abstract
Metal pollution is a global problem growing at an alarming rate and aquatic organisms are continuously exposed to elevated levels of metals seriously threatening the whole ecosystem. The present study was undertaken to assess bioaccumulation of cadmium in the fish, *Clarias batrachus* (Linn.) and to address its correlative impacts. Heavy metal salt viz. cadmium chloride of analytical reagent grade was used to conduct the toxicological experiments. Experimental fishes of both genders were divided into four groups. Each group was kept in concrete tank containing 30L of water separately, at a density of 20 fish / tank in replicate. Group I was kept as control, group II, III, and IV were exposed to 0.05ppm, 0.5 ppm and 1ppm dose of $CdCl_2$ respectively. Calorimetric analysis was carried out by Lowry's Method [19], while statistical analysis was carried out by using ANOVA. As a result, we found that $CdCl_2$ halts the metabolism in fishes exposed to different concentration of $CdCl_2$. It was observed that the control sample has 69.26 µg of protein per ml. The protein content was high in controlled fish and diminishes in order of increasing ppm of $CdCl_2$. From the current study, it was concluded that the controlled fish was having highest protein than the experimental fish, which were exposed to Cadmium chloride at different concentrations. So, the Cadmium chloride affects the overall protein metabolism because the toxic effects of cadmium chloride on muscle protein resulted in decrease in protein levels, which can provide insight of biochemical analysis in future perspectives.

Keywords: Biochemical changes, cadmium, *Clarias batrachus*, fresh water cat fish

1. Introduction
Clarias batrachus, the walking catfish, locally named as "Magur" is broadly distributed throughout the Indian sub-continent. *Clarias batrachus* is a bottom feeder with nocturnal habits. The diversity of catfishes is highest in Africa. Many of the species are of great importance in both fisheries and fish culture. There are currently 60 species recognized in this genus. Due to its fast growth and high marketability, it is mostly cultured either alone or in conjunction with other catfish species [1]. Information on the nutrition of this species is limited to its protein, energy and protein maintenance. In general, cadmium is a biologically non-essential, non-biodegradable, persistent heavy metal and its compounds are known to have high toxic potentials. Further, continuous, low level cadmium exposure may have a gross biological impact comparable to that of recurring exposures of much greater intensity. In fresh water fish, cadmium uptake is taking place mainly through three routes namely, gills, skin and also from food via the intestinal wall [2].
Fish have the ability to accumulate heavy metal in their tissues by the absorption along the gill surface and gut tract wall to higher levels than the toxic concentration in their environment [3]. Metal pollution is a global problem growing at an alarming rate and aquatic organisms are continuously exposed to elevated levels of metals seriously threatening the whole ecosystem. Heavy metals are normal constituents of marine environment that occur as a result of pollution principally due to the discharge of untreated wastes into rivers by many industries [4, 5]. Under natural exposure conditions, prediction of toxic effects based on environmental or tissues concentrations remains difficult while many studies have examined the relationship between metal exposure, accumulation and toxicity under laboratory conditions [6, 7].

Furthermore, fish and seafood are one of the main links between the heavy metal present in the environment and the human exposure [8, 9, 10]. Vetillard and Bailhache, showed that Cd levels found in liver follow the predictive linear pattern of metal accumulation in this tissue. Cd not only accumulated in the liver but was also increased in the brain, even if levels were far lower than those found in the liver. Accumulation in the brain seems to be dependent on the administration route [11]. Bioaccumulation of heavy metals in tissues of aquatic organisms has been recognized as an subsidiary measure of the abundance and availability of metals in the aquatic environment exhibited the occurrence of heavy metals in tissues of fish [12].

In current study, a fresh water catfish *Clarias batrachus* (walking catfish) was selected as the model organism due to its fast growth and high marketability. Nutrition of this species is limited to its protein, energy and protein maintenance requirements. Moreover, due to presence of accessory respiratory organs catfish can survive in waters with low oxygen concentration and high temperature.

Mean values of cadmium in the blood and urine of the U.S. population were reported in the National Health and Nutrition Examination Survey (NHANES) 1999–2008. Blood cadmium tends to reflect recent exposures and urinary cadmium reflects cumulative cadmium exposure and body burden (particularly, kidney cadmium levels). Cadmium very much affects the energy metabolism, which in long term cause the death of the individual organism and affects the whole community [13]. Accelerated release of heavy metals into the aquatic environment leads to serious water pollution problems, persistence and bioaccumulation. Therefore, it is important to know the various sources of discharge of heavy metals into aquatic ecosystems throughout the world.

1.1 Aim of the study

The proposed study was planned to investigate the changes in biochemical parameters i.e. protein concentration in *Clarias batrachus*. Specific aims of the current study were:

- To observe the toxicity of cadmium in fish
- To analyze the protein comparison in fishes exposed to CdCl2
- To study the effect of CdCl2 on overall growth of fishes.

2. Materials and Method

2.1 Fish collection

Clarias batrachus with an average weight of normal size ranging between 14 cm to 20 cm in length and their weight was between 250 gm to 400 gm (Fig 1, 2, 3, 4). The fishes were transported from the Farm in oxygenated bags to the laboratory and immediately transferred into glass aquaria of 30L capacity containing well aerated, unchlorinated ground water. The water was discharged every day. The healthy fish that showed active movement were only used for the experimentation. Fishes were feed with fish Food.

Fig 1: *Clarias batrachus* (Experimental organism)

Fig 2: *Clarias batrachus* in aquarium in laboratory at Dept. of Zoology D.I.B.N.S. ($CdCl_2$ conc. In aquarium is 0.5 ppm)

Fig 3: *Clarias batrachus* in aquarium containing $CdCl_2$ concentration of 1 ppm

Fig 4: *Clarias batrachus* in aquarium containing $CdCl_2$ concentration of 0.05 ppm

2.2 Study Design

Experimental fishes of both genders were divided into four groups. Each group was kept in concrete tank containing 30L of water separately, at a density of 20 fish / tank in replicate. Group I was kept as control, group II, III, and IV were exposed to 0.05ppm, 0.5 ppm and 1ppm dose of $CdCl_2$. The

fishes were not fed during these days and water was changed at interval of 10 days and making the cadmium chloride concentration same as were before. The fishes were kept under observation for 30 days in Cadmium containing water. The effect of Cd concentrations on muscle protein was investigated after 30 days.

2.3 Xenobiotic used in the study
Lethality of fishes was estimated by $CdCl_2$.

2.4 Protein Estimation by Lowry's Method
Proteins are the most abundant compounds in the serum and are used in forming defensive molecules that helps the body to fight against infections. Lowry's Method is highly sensitive and can detect protein levels as low as 5µg/ml. Lowry's assay for total protein is one of the most commonly performed colorimetric assays [19]. This procedure is sensitive because it employs two color-forming reactions. It uses the Biuret reactions in which Cu^{2+} in presence of a base reacts with a peptide bond of protein under alkaline conditions resulting in reduction of cupric ions (Cu2+) to cuprous ions (Cu+), and Lowry's reaction in which the Folin Ciocaltaeu reagent which contains phosphomolybdic complex which is a mixture of sodium tungstate, sodium molybdate and phosphate, along with copper sulphate solution and the protein, a blue purple color is produced which can be assessed by measuring the absorbance at 650-700nm.

2.5 Preparation of Standard Protein Solution
Total protein content was estimated by using Bovine Serum Albumin (BSA) This solution is prepared to derive standard curve, which is used to measure protein concentration in sample by comparing this with sample.

- Weigh 0.05g of BSA and add it to 500 ml volumetric flask containing distilled water.
- The final Stock solution is having protein content of 100 µg /ml.

2.5.1 Protein Sample
The fish were taken out of Aquaria and were met with death either by decapitating or by formaldehyde treatment. After that the sample of muscles were cut of from fish and were cleaned with tap water. The samples were deep freeze for 24 hours. Deep freezing ensures that proteins in sample do not degrade and low temperatures inhibit bacterial growth. Before extraction the muscles were homogenized in homogenizer or by manual mechanical methods. Protein was extracted by treating homogenized mixture with Methanol/Chloroform separation method.

2.5.2 Method for Standard Protein sample
Three aliquots of protein sample having concentrations (0.4, 0.6, 0.8) ml/mg were taken in test tubes to measure the absorbance. 1ml distilled water and 5ml of Reagent C i.e. Alkaline Copper Reagent (Lowry Reagent) was added to all test tubes and Incubated for 10 mins then 0.5 ml of Folin Ciocalteu's Phenol Reagent was added to all samples and after 30 mins of incubation Absorbance was measured by using spectrophotometer at 650 nm. Reagent C was added in all protein samples i.e 20 µg, 40 µg, 60 µg and 80 µg conc. and was followed by adding Follins Reagent before Spectroscopy. Absorbances were measured for all concentrations and were noted down. Using the absorbance data and the known protein concentration the Standard curve was plotted.

3. Calculations
The absorbance's of protein samples on spectrophotometer were measured for 3 different samples containing 0.8, 0.6, 0.4 ml/gm of protein respectively.
The different known concentrations were made for BSA and their absorbance was measured and a graph was plotted which derives the equation y= mx from which the calculation can be done for unknown samples.
Graph absorbance versus Concentration obtains the equation of line
y=mx

3.1 Statistical Analysis
One-way (ANOVA) was used to compare protein concentrations in different samples from three variables (three samples). Differences amongst means were determined using Duncan's Multiple Range Test (DMRT). Standard deviation (SD) was calculated. Significance level was set at P = 0.05 confidence limit. All statistical data was calculated by SPSS version 18.0.

4. Results
The chemical composition of protein is usually used as an indicator of the nutritive value as well as the physiological condition of fish and its habitat [14, 15]. Status of the fish selected for the current study is shown below in table 2.

Table 1: Standard Solution of BSA and their protein concentration. (Dilutions for Standard)

Volume of Distilled water.	Volume of Stock BSA Sol.	Final Concentration of Protein
0.0 ml	1.0 ml	100 µg/ml
0.2 ml	0.8 ml	80 µg/ml
0.4 ml	0.6 ml	60 µg/ml
0.6 ml	0.4 ml	40 µg/ml
0.8 ml	0.2 ml	20 µg/ml
1.0 ml	0.0 ml	0.0 µg/ml

Table 2: Status of fishes at different chemical concentrations

	Fish 1	Fish 2	Fish 3	Fish 4
Chemical Concentration of $CdCl_2$	Control	0.05 ppm	0.5 ppm	1.0 ppm
Status after 30 days	Alive	Alive	Alive	Dead after 28 days

Table 3: Absorbance's at 650 nm by BSA samples

BSA Protein µg/ml	Absorbance's at 650 nm
0 µg/ml	0.000
20 µg/ml	0.090
40 µg/ml	0.170
60 µg/ml	0.250
80 µg/ml	0.330
100 µg/ml	0.409

Table 4: Protein Concentration and absorbance of samples from Controlled and Experimental Aquaria

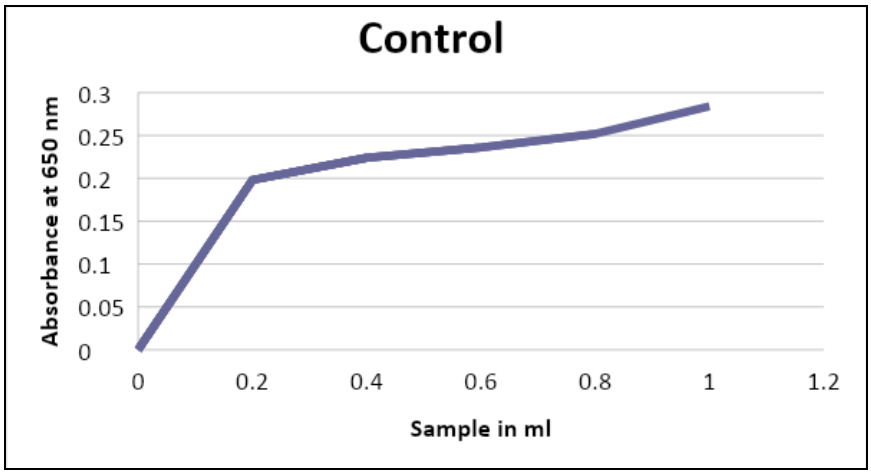
Sample	Absorbance (650 nm)			X= y/m=y/0.004			ProteinIn Micrograms		
	Controlled	0.05ppm	0.5ppm	Controlled	0.05ppm	0.5ppm	Controlled	0.05ppm	0.5ppm
0.2 ml	0.198	0.186	0.184	0.198/0.0041	0.186/0.0041	0.184/0.0041	48.29µg/ml	45.36µg/ml	44.87 g/ml
0.4 ml	0.224	0.221	0.202	0.224/0.0041	0.221/0.0041	0.202/0.0041	54.63µg/ml	53.90µg/ml	49.26µg/ml
0.6ml	0.236	0.234	0.206	0.236/0.0041	0.234/0.0041	0.206/0.0041	57.56µg/ml	57.07µg/ml	50.24µg/ml
0.8 ml	0.252	0.247	0.215	0.252/0.0041	0.247/0.0041	0.215/0.0041	61.46µg/ml	60.24µg/ml	52.43µg/ml
1.0 ml	0.284	0.274	0.234	0.284/0.0041	0.274/0.0041	0.234/0.0041	69.26µg/ml	66.82µg/ml	57.07µg/ml

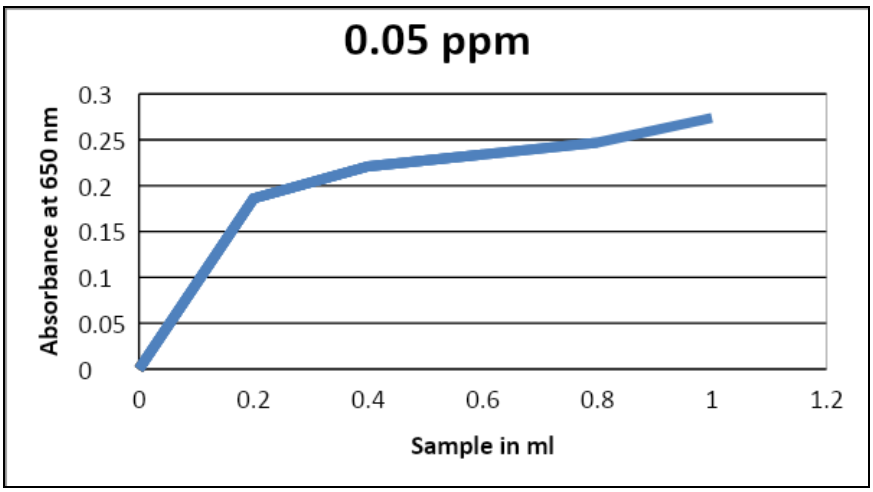
In other sample, which is from the aquaria having 0.05 ppm $CdCl_2$, the protein content is 66.82 microgram proteins per ml, which is slightly lower than the controlled fish. When protein sample is observed from aquaria containing 0.5 ppm $CdCl_2$ we come to know that this has got least protein concentration of all samples. This sample calculates 57.07 µg of proteins per ml as shown in table 4.

Table 5: Absorbance's at 650 nm of Protein sample (Control)

		Lowry Reagent (Reagent C)			Follins Reagent			Absorbance at 650nm		
Protein Sample	Distilled water	Control	0.05ppm	0.5ppm	Control	0.05ppm	0.5ppm	Control	0.05ppm	0.5ppm
0.2 ml	0.8 ml	5 ml	5 ml	5 ml	0.5 ml	0.5 ml	0.5 ml	0.198	0.186	0.184
0.4 ml	0.6 ml	5 ml	5 ml	5 ml	0.5 ml	0.5 ml	0.5 ml	0.224	0.221	0.202
0.6ml	0.4 ml	5 ml	5 ml	5 ml	0.5 ml	0.5 ml	0.5 ml	0.236	0.234	0.206
0.8 ml	0.2 ml	5 ml	5 ml	5 ml	0.5 ml	0.5 ml	0.5 ml	0.252	0.247	0.215
1.0 ml	0.0 ml	5 ml	5 ml	5 ml	0.5 ml	0.5 ml	0.5 ml	0.284	0.274	0.234

Three fish in three different aquaria having the 0.0 ppm (Controlled), 0.05 ppm and 0.5 ppm of cadmium chloride resulted in different protein concentrations in their samples when processed through Lowry method [19].





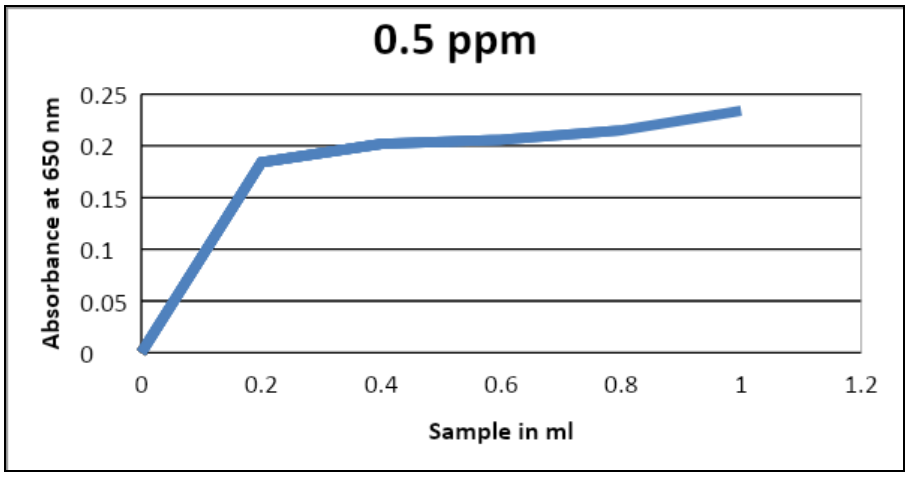
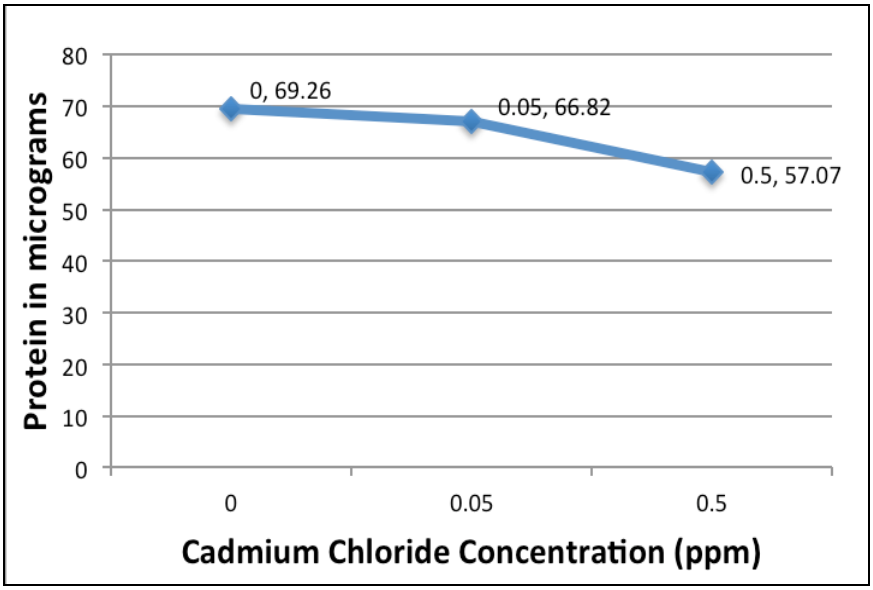
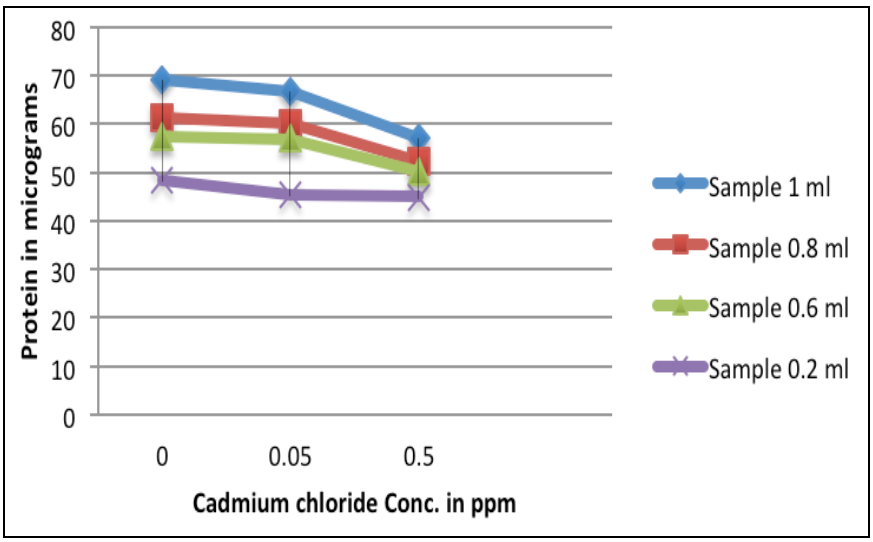


Fig 5: Graphs representing the absorbance versus sample concentration in three different samples from control and exposed fishes.

4.1 Protein Estimation

Equation of Line derived from Standard Curve graph for BSA calculates the concentration of protein sample. The equation is y =mx.





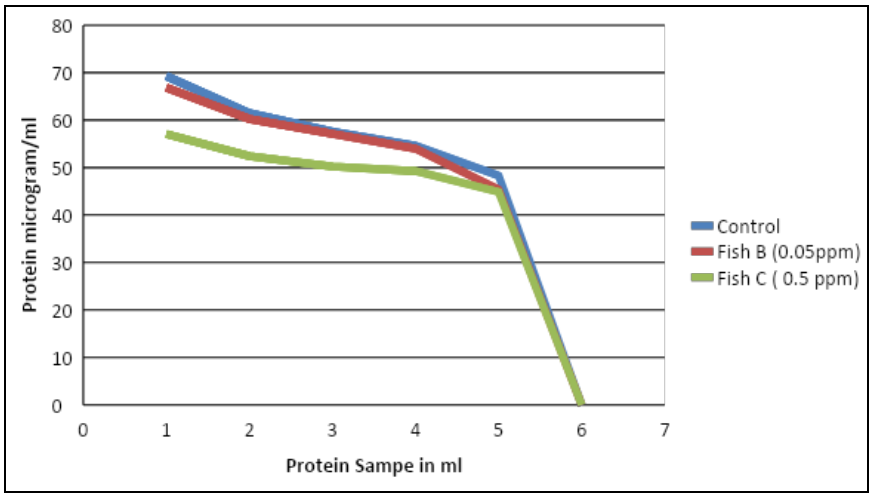


Fig 6: Graph representing the protein concentration in three experimental fish

Fig 6: Graph showing Protein concentration at different levels of $CdCl_2$ concentration. The protein concentration comparison has been shown in above figure. Three lines shown in the graph represent the individual concentrations of proteins for three different samples from aquaria containing 0.0 ppm (Controlled), 0.05 ppm and 0.5 ppm of $CdCl_2$ respectively.

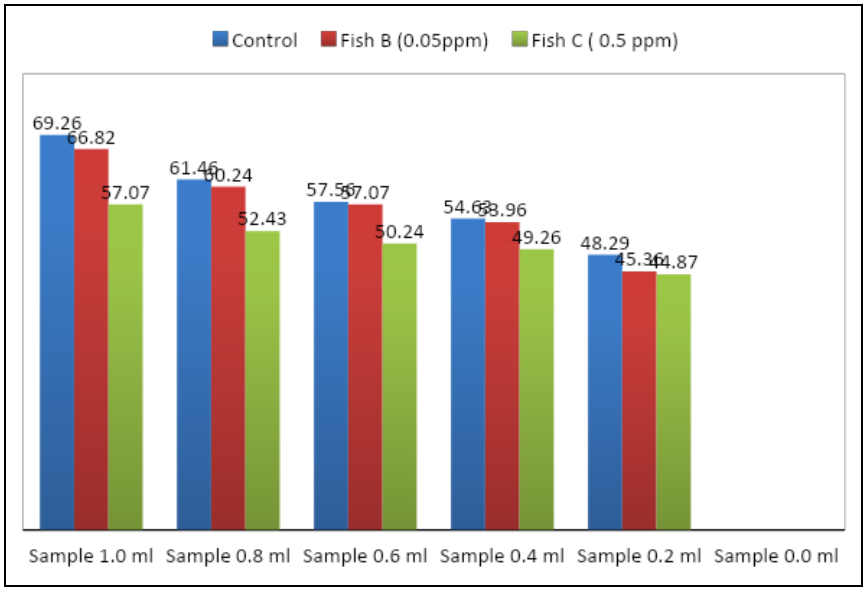


Fig 7: The bar diagram depicts the individual protein concentration in five samples. Blue, Red and Green bars represent 0.00ppm, 0.05ppm and 0.5ppm respectively.

- The Blue bar i.e Controlled shows the highest concentration of proteins in sample.
- The Red bar shows slightly lower concentrations of protein in sample than the controlled one.
- The Green bar shows the least concentrations of proteins to the controlled one.

Table 6: Statistical analysis for Protein concentration

Statistical Analysis for Protein Concentration				
	Standard	**Control**	**Exposed to 0.05 ppm**	**Exposed to 0.5 ppm**
1	100	69.26	66.82	57.07
2	80	61.46	60.24	52.43
3	60	57.56	57.07	50.24
4	40	54.63	53.96	49.26
5	20	48.29	45.36	44.87
N	5	5	5	5
X	60.000	58.240	56.690	50.774
S	31.623	7.815	7.924	4.467
$\mathbf{X}_{avg}$	56.426			

Sum of Squares: 240.394
Mean of Squares: 80.131
F statistics: 0.2802, degree of freedom = 3
P-value: $p < 0.1$
Total Sum of Squares: 4815.702

Table 7: Protein-concentration comparisons in three protein samples from Controlled Aquaria, Aquaria 2(0.05ppm) and Aquaria 3 (0.5ppm)

Protein Samples in ml	Protein Concentration		
	Fish A Control Fish	Fish B 0.05 ppm ($CdCl_2$)	Fish C 0.5 ppm ($CdCl_2$)
	Protein µg/ml	Protein µg/ml	Protein µg/ml
Sample 1.0 ml	69.26	66.82	57.07
Sample 0.8 ml	61.46	60.24	52.43
Sample 0.6 ml	57.56	57.07	50.24
Sample 0.4 ml	54.63	53.96	49.26
Sample 0.2 ml	48.29	45.36	44.87
Sample 0.0 ml	0.00	0.00	0.00

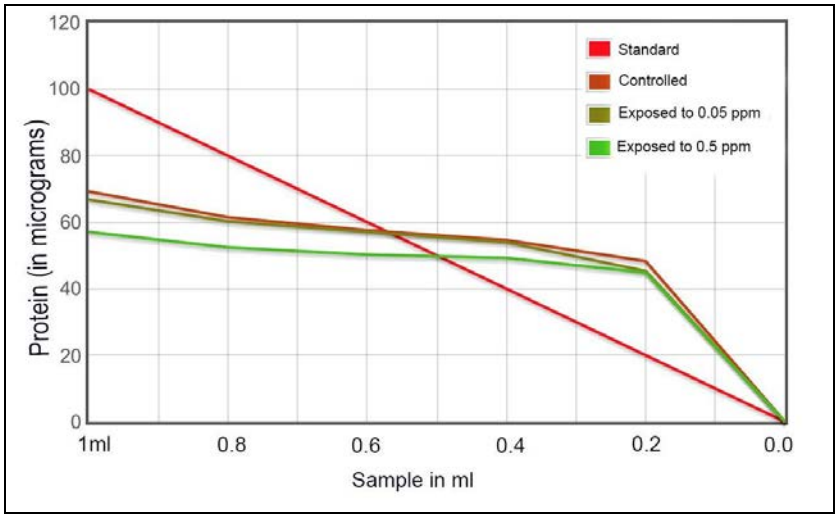


Fig 7: Represents the graph derived from statistical analysis.

In graph all the samples have been analyzed including the standard BSA sample, which the red line represents in the graph. The red line in the graph is standard curve, which comes to be straight line from which the comparison has been made between the samples from fishes.

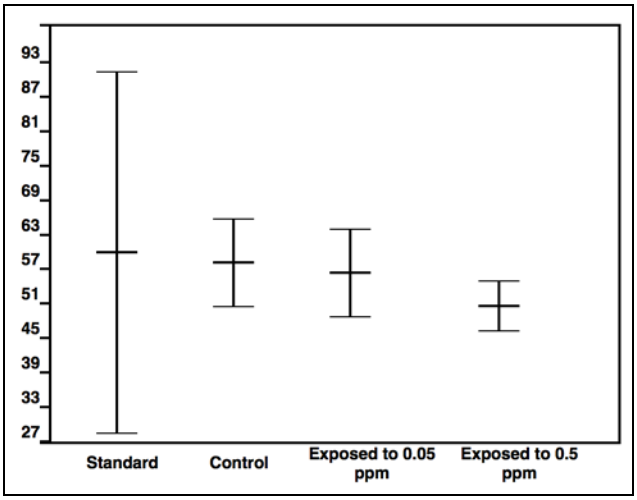


Fig 8: Graphs derived from ANOVO (statistical analysis)

5. Discussion

Water pollution by organic and inorganic sources has been identified as one of the most significant factors in the intoxication of marine organisms, one of them is fish [16]. Fish is one of the foremost protein sources for humans that play a role in reducing the blood cholesterol level and offers omega-3 fatty acids that diminish the danger of stroke and heart related disorders [17]. Of all aquatic species, fish are predominantly sensitive to waterborne contamination and are documented as bio-indicators for water quality monitoring. To reduce the negative effects of Reactive Oxygen Species (ROS), fish possess an antioxidant defense system like other vertebrates that utilizes enzymatic and non-enzymatic mechanisms. The use of fish in environmental monitoring has become increasingly important in recent years in the investigation of natural variability, as well as anthropogenic substances, many of which function as prooxidants, accumulating in aquatic environments [18]. Proteins are extremely sensitive to heavy metal intoxication [20]. In current study, the toxic effects of Cadmium chloride on muscle protein of *Clarias batrachus* were observed after protein estimation of all the samples. The Fig.1 shows our Experimental organism, which is the *Clarias batrachus*. The *Clarias batrachus.* The fish is acclimatized in the current conditions of Aquarium. Now in the next figure (Fig 2) shows us the *Clarias batrachus* in aquarium in laboratory at Dept. of Zoology D.I.B.N.S. $CdCl_2$ concentration in aquarium 0.5ppm. Furthermore, we have fig 3, fig 4, which shows *Clarias batrachus* in aquarium containing $CdCl_2$ concentration of 1 ppm and 0.05 ppm respectively.

As the fishes were subjected to cadmium, we found that all fishes stay alive except the one aquarium fishes which were subjected to 1 ppm Cadmium chloride for a period of 28 days as shown in table 2.

The results were different for all samples but the controlled one and the least ppm of $CdCl_2$ containing aquaria were having slight difference as shown in the Table 6. The protein content was high in controlled fish and diminishes in order of increasing ppm of $CdCl_2$. It was concluded that the control sample has 69.26 µg of protein per ml i.e. no cadmium chloride was added in it, it was having normal conditions. In Table 5 the comparison of Protein concentration in samples have been shown using the lowery and follins reagent which

showed that the highest protein concentration was present in controlled samples and lowest in those samples which were exposed to 0.5 ppm, followed by 0.05 ppm. In the same way when different protein samples of different concentrations are measured for protein estimation, the result comes to be same. The result from all samples follows the same trend that is the highest protein concentration is measured in controlled fish and the least protein concentration was measured in that aquarium which was having highest $CdCl_2$ concentration as shown in table 7. ANOVA results also showed that the fishes which were exposed to $CdCl_2$ i.e. 0.05 and 0.5 ppm resulted in low protein quantities as compared to control fishes, All the results were compared with standard as shown in figure 8. The results observed in this study explains that the $CdCl_2$ halts the metabolism in fishes exposed to different concentration of $CdCl_2$. It was observed that the fish which was exposed to high levels of $CdCl_2$ have less protein concentration in their muscles than the controlled one. On comparing this study with other studies, the results were the same and proved that the high concentration of $CdCl_2$ decreases the protein content in muscle of fish.

Conflict of interest

Authors have no conflict of interest.

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