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Bioaccumulation and response of Metallothionein in the grey mullet fish *Mugil cephalus* exposed to Lead

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Abstract
A laboratory experiment was conducted to determine the lead acetate accumulation in *mugil cephalus*, and also studied the Metallothioneins bioaccumulation in all tissues, except muscle, was consistently time- and dose-dependent. The accumulation of lead for 4 weeks exposure was in the following order Gill>Liver> Muscle. An independent relation was observed between accumulation factors (AFs) and exposure concentration. In the liver and gill, Accumulation was differed from the exposure duration at a high lead concentration at 2 and 4 weeks ($P < 0.05$). This study indicated that the gills were as sensitive as the liver to lead toxicity. This study aimed to verify Metallothioneins are present in fish tissues, if they can be used as biomarkers for metal exposure and to characterize the metal speciation present in the different organs of the species. Among the various methods are there to verify MT presence in fish tissues were conducted: 1D/2D gel electrophoresis was followed to observe the presence of metallothioneins proteins in fish tissues. However the accumulation of lead in the fish tissues may elevate the Metallothioneins by increased level of lead uptake in grey mullet *mugil cephalus.*

Keywords: Lead, Bioaccumulation, Metallothionein, Mullet fish, *Mugil cephalus*

1. Introduction
Substantial metals are viewed as major anthropogenic poisons in oceanic conditions, as they represent a genuine risk to amphibian creatures because of their poisonous quality, diligence, and bioaccumulation propensities [1]. Metal sullying of the oceanic condition is a long haul issue, since metals amass in amphibian life forms, including fish, and persevere in silt [2]. Fish are a significant wellspring of human nourishment, and those from metal-debased locales present a potential hazard to human wellbeing. Sea-going living beings present, basic wellsprings of amassed metal as well as can cooperate with metals, adjusting their poisonous quality. Because of the work of the biosphere with metals, life forms have created different systems to ensure themselves against antagonistic impacts of these particles and their mixes. The homeostasis of metals in both plant and creature cells keep up by low-sub-atomic mass mixes rich in-SH moieties. In creatures, metallothioneins (MT) assume a key job in the keeping up of metal homeostasis. Metallothioneins are a gathering of low atomic mass (2 to 16 kDa) single-chain proteins. The metal-restricting space of MTs comprises of 20 cysteine deposits compared with fundamental amino acids (lysine and arginine) organized in two thiol-rich destinations [3]. Based on their proclivity to metals these proteins can ship basic metals to place of need or detoxify harmful metals to secure cells [4]. Metallothionein synthesis varies with fish species, age and an analyzed tissue [5]. External factors, such as season, temperature, and diet, can also effect MT induction [6]. The aim of the present study was to determine the metal Content and to assess the effect of metals on metallothionein levels in fish *mugil cephalus* tissue under experimental conditions.

2. Materials and Methods
The present study conducted during the month of September-October 2018 by the Department of Marine Biology, Vikrama Simhapuri University, Nellore, Andhra Pradesh, India.

2.1 Study Area
The Nellore district has 169 km of the coastal line with habitation of the fishermen community along the east coast of India. The Nellore coastal region is suitable for the brackish water aquaculture farming, which recently gradually increased some industrial activities. The present sampling sites of Krishnapatnam coastal village have shipping activities also nearly the presence of some thermal power plants from this coastal region Fig 1. This sampling station is one of the major marine fishing areas which accomplished by the fishermen community along the coast. Considering all the factors, samples collected from Krishnapatnam for the present study.

2.2 Fish collection
Brackish water fish flathead grey mullet *(Mugil cephalus)* ranging from 10-14 cm in length and weighing between 110-125g were collected from coast of Krishnapatnam coastal village, Nellore district of Andhra Pradesh (Fig 1). The collected fish samples were carried to the laboratory with an artificial aeration system.

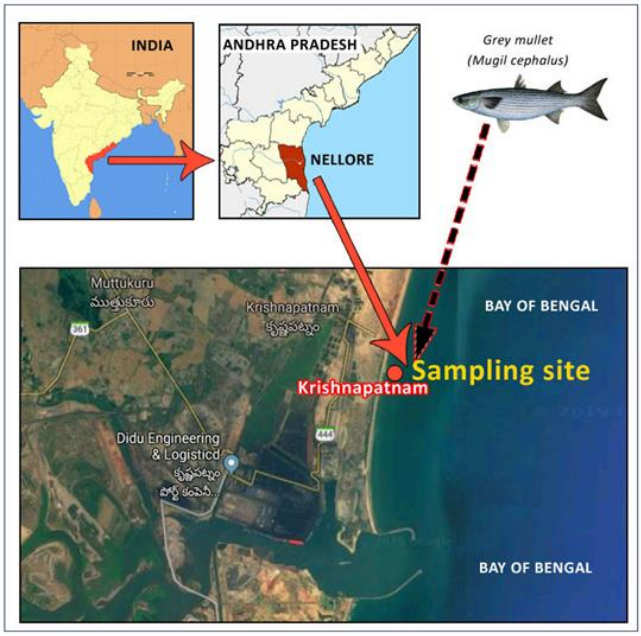

Fig 1: Map showing the sampling site Krishnapatnam beach of Nellore coast.

2.3 Fish conditioning for experiment

The fishes were acclimatized to the laboratory conditions for 5 days with 15ppt (ppt-parts per thousand) salinity and maintained the water temperature at 26±8ºC. The water exchanged (10%) in every 2 days and they were fed with formulated fish feed (2% body weight) and removed uneaten feed, waste materials from the experimental tanks (22 liters). The water quality parameters like, dissolved oxygen, salinity, pH were maintained constantly in both control and experimental tanks.

2.4 Behavior observation

The behavioral changes of the fish before and after exposure to the toxic metal Lead (Pb) was monitored. Physiological responses like rapid opercular movement and frequent gulping of air were observed during the initial stages of exposure after which it became occasional. The dead fish were removed from the tank, when fish mortality observed during the experiment.

2.5 Acute toxicity test

According to results obtained from the bioaccumulation toxicity test were carried out in order to calculate the 2-4 weeks for lead acetate accumulation for bioaccumulation toxicity tests were [25, 50, 100, 200 and 400 ppm]. The present investigation as bioaccumulation concentration, the observation on fish mortality were recorded after 2weeks intervals and dead fish were taken out from the experimental tanks and they were slightly blotted dry.

2.6 Heavy metal analysis by Atomic Absorption Spectroscopy

AAS is most widely using an analytical technique for the determination of trace and heavy metals up to parts per billion levels. AAS is a very useful technique to determine trace levels of multi-elements in single aspiration. Dead fish obtained from acute toxicity trials were disserted and their tissue samples viz., gills, liver and muscle were separated for heavy metals analyses, the tissue samples were rinsed with distilled water and blotted with blotting paper. They were digested in HNO_3 and $HClO_4$ (3:1 V/V) by placing in the flasks on the hot plate until a clear solution was obtained (S.M.E.W.W., APHA). After this digestion, samples were cooled, diluted, filtered and then determined for metal concentration by using the Atomic Absorption Spectrophotometer (Analyst-400 Perkin Elmer, USA). Calibration standards for metal were made by the serially diluting stock solutions with reagent grade distilled water and determined standards were run along with samples. Normal single hollow cathode lamps were used for irradiation followed by APHA [7].

Table 1: Atomic Absorption Spectroscopy Condition

AAS Parameters	Condition
Time of Measurement	62 Seconds
Wave length	251.7 nm
Lamp current	4.0 mA
Spectral width slit	0.7 nm
Flame air	15.0 L/ min
Acetylene flow rate	2.0 L/min

2.7 Sample processing

MT extraction in fish bile followed the protocol proposed by Erk *et al.* (2002) [8] specifically for this protein, using heat treatment. Briefly, bile samples were thawed and homogenized at a 3:1 ratio in sterile eppendorfs in a solution containing Tris-HCl 20 mM pH 8.6, PMSF (phenyl methyl sulphonyl fluoride) 0.5 mM as an anti proteolytic agent and β-mercapto ethanol 0.01% as a reducing agent. They were then centrifuged at 20,000×g for 1 h at 4°C. The supernatants were then carefully separated from the pellet and transferred to new sterile eppendorfs and heated at 70°C for 10 min. Another centrifugation step was conducted at 20,000×g for 30 min at 4°C and the final supernatants containing the MTs were separated and frozen at-80° C until analysis.

2.8 SDS-PAGE analysis

All out protein content was measured by the Lowry strategy. Two sorts of 1D partitions were directed, on 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and on 16.5% low sub-atomic weight SDS-PAGE gels, following the convention of Yan *et al.* (2000) [9] .which utilizes trycine rather than glycine as a trailing particle so as to all the more proficiently separate little proteins (<10 kDa). Three recreates of each example (12-g in every path) were directed, to guarantee technique reproducibility. After 1D detachment, MTs were likewise measured by densitometry, in which a standard bend was plotted by running known convergences of a Bovine Albumin Serum (BSA) standard and tallying the pixels for each point by utilization of imaging programming (Adobe Photoshop 7.0 ®). The metallothioneins groups were then evaluated by correlation with this standard bend. Gels were recolored both by silver recoloring pursued by Lopez *et*

al. 2000 [10] with minor changes and by Coomassie Blue G-250. Three recreates were made of every individual example, to guarantee reproducibility. The 2D partition was likewise led, in which tests were run on both the 15% Tris-Glycine convention and the 16.5% Tris-Tricine convention. Tests (8-g per test) were pictured at a 3-10 pH extend. Isoelectric concentrating was led on an isoelectric centering unit. The atomic loads of the protein groups and spots were resolved utilizing Biorad's Precision Plus ProteinTM Dual Color Standards. Gels were examined utilizing an Image Scanner II (GE Healthcare, Uppsala, Sweden) with the densitometer working at 300 dpi goals. Picture Master 2D Platinum 6.0 programming (GeneBio,Geneva, Switzerland) was utilized for gel imaging examination.

3. Results and discussion

Lead accumulation in the organs of the mullet depending on the exposure time and exposure dose are shown in Table. 2.

Table 2: Accumulation factor (AF) over time in gill, liver and muscle tissues of grey mullet, *M. cephalus* (mean ±S.D.), exposed to the different concentrations

Tissue	Lead acetate (gg L-1)				
	25	**50**	**100**	**200**	**400**
2 weeks exposure					
Gill	0.15±0.02	0.19±0.03	0.74±0.02	2.06±0.04	2.78±0.29
Liver	3.42±0.86	2.78±0.39	2.04±0.49	1.86±0.31	1.55±0.24
Muscle	0.29±0.04	0.47±0.07	0.41±0.17	0.09±0.02	0.38±0.03
4 weeks exposure					
Gill	0.57±0.16	10.69±0.22	7.68±0.49	4.59±0.19	3.92±0.38
Liver	3.59±0.82	4.31±0.25	2.13±0.51	2.41±0.11	2.39±0.37
Muscle	0.07±0.0.2	1.86±0.03	1.42±0.0.7	0.51±0.02	0.48±0.03

As prove, the Hg aggregation brought about a net increment in the complete Hg content in all organs aside from the muscle. At 400 µg L-1, the gill, liver, and muscle demonstrated most extreme Hg content; nonetheless, these three tissues of the fish measurably varied to one another. The collection examples of Hg following 4-week introduction happened in the accompanying request: Gill ≈ liver ≈ muscle. Metal gathering in the organs of fish is needy upon the presentation time and introduction portion just as different elements, for example, temperature, age, association with different metals, water science, and metabolic movement of the fish [11].

Here, Hg gathering in the mullet Liver was higher than that muscle and gill at 400 µg L-1, demonstrating that Hg collection in the Liver was more compelling than that in the gill and muscle of the mullet (Fig.2). The collection of metal in the liver was higher than that of different tissues during the interminable lead introduction. The principle area of metal aggregation fluctuates unequivocally crosswise over fish species. Furthermore, the support of high amassing in gills and liver has regularly been watched, as these tissues involve the chief course of discharge for most toxicants. In the present investigation, gill tissue contained a considerable measure of Hg during the exploratory period (Table. 2). Have demonstrated that the gills are exceptionally gathering organs in fish because of their cozy association with the outer condition [12]. Then again, the convergences of Hg were lower in the muscles contrasted with different organs analyzed in this investigation. This outcome is especially significant in light of the fact that the muscles add to the best mass of the tissue that is expended as nourishment. (Table.2 and Fig.2) presents the AFs for different organs after Hg presentation in the mullet. The AFs expanded with the introduction time frame and were contrarily identified with the presentation focus in the organs of the mullet. The AFs were determined for two significant purposes: first, to quantify the amount Hg is amassed as for fluid introduction fixation, and second, to decide as far as possible in the capacity of fish to aggregate metals [13].

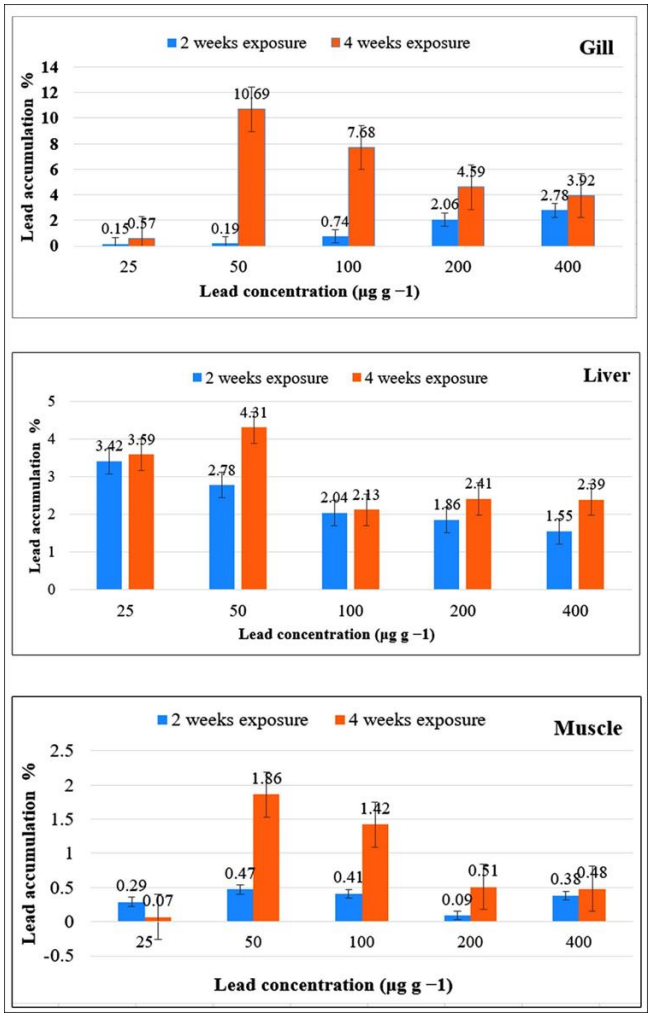

Fig 2: Accumulation of Lead (Hg) in the selected organ tissues of mullet; *M. cephalus* exposed to the different concentrations of Hg.

3.1 Metallothionein by SDS - PAGE method

Subjective Laemmli 1D electrophoresis indicated the nearness of protein groups in the scope of 12-13 kDa. Low sub-atomic weight SDS-PAGE gels indicated a similar 12 kDa groups (Fig. 1), subsequently showing that it is conceivable to acquire equivalent results utilizing the two kinds of SDS-PAGE since 14 kDa protein groups are not all that little (<10 kDa) as to not be envisioned on Laemmli frameworks. Accordingly, the Laemmli framework was picked for all electrophoresis runs. MT measurement test was seen in the gels: tests demonstrated

incredibly light to semi-dull groups, at times not obvious by any means, while the examples someplace indicated middle person, noticeable groups. Tests from the introduction analysis to Hg demonstrated the most unmistakable and communicated groups, showing increasingly 14 kDa protein articulation in this circumstance (Fig. 3) reliable with raised metal sullying present in this investigation. The paths (gel paths 1-7) in this figure are tests of a similar fish mullet *M.cephalus*, defiled with Hg separately, indicating the force contrasts at different fixations, in this way affirming metallothionein nearness in the tissues of the fish, and similar articulation conduct in various ecological circumstances. Protein measurement by densitometry is a generally straightforward technique, where the gel is checked and afterward broke down through picture programming (Adobe Photoshop®). The band forces of the gauges (in pixel check) are then determined to plot an investigative bend and afterward measure the groups of premium (Fig. 3). we are sure about the ID of the SDS-PAGE protein groups as metallothioneins, even by just utilizing their atomic load for distinguishing proof Also, in the fish tissue tests the main low sub-atomic groups present demonstrated sub-atomic load of 12 kDa, while some other groups indicated sub-atomic load as high as 50 kDa, barring their recognizable proof as MT, and the Ellman spectrophotometric response, explicit in the identification of cysteine-rich proteins, in which metallothioneins are incorporated (examined underneath), indicated a similar pattern as the 1D SDS-PAGE gels.

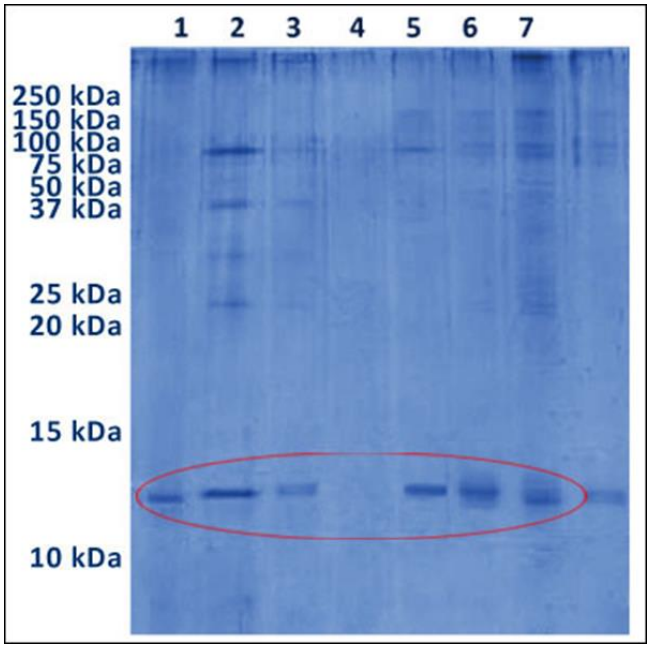

Fig 3: Gel showing the different metallothionein band intensities for environmental and control samples, bands1-6 showing fish samples Gill, Liver and Muscle of *M. cephalus*. Band 7 showing molecular weight standard for MT.

Thus, the fact that we conducted a specific thermal extraction, which would leave very few other proteins in addition to MTs, gives an extremely high certainty that the protein bands present in the gels are metallothioneins. SDS-PAGE Gel Electrophoresis shows a more detailed expression of the proteins present in the bands, and also indicates several differences in the MT profile, as seen in Fig. 3. These protein spots are also all in the range of 12 kDa, further confirming their identity as MT.

4. Conclusions

In conclusion, results from these studies indicate that a accumulation of heavy metals in fish tissues was done by experimental exposure by assessing Atomic Absorption Spectoscopy high fixation in Liver, Gill, and muscle of the M.cephalus with reference to the amassing contemplates the examples were performed SDS-PAGE Gel electrophoresis concentrate to demonstrate that metallothionein is available in tests. In this technique, the nearness of this metal-restricting protein was checked. We were seen that the groups saw in the SDS-PAGE examinations are metallothionein groups since MT in M.ceehalus has been portrayed as displaying a 12 kDa atomic weight (in the liver), and other fish species MT have been depicted as differing from 10 to 13 kDa. Additionally, different reasons demonstrate this is the situation: in a few of the examples, the main low sub-atomic groups present were the 12 kDa groups, while different groups were especially out of the portrayed MT scope of under 20 kDa. This is normal since we led a particular warm extraction, which would leave not many different proteins aside from MTs since relatively few proteins are heat stable. The Ellman spectrophotometric response is explicit in the discovery of cysteine-rich proteins, a gathering that incorporates MT, and has been utilized widely in MT measurement. This strategy indicated a similar pattern saw in the SD-PAGE gels, in which MT from metal uncovered living beings was increasingly communicated. Various focuses from two diverse introduction timespan were watched for *M.cephalus*: tests acquired from the indoor test, from the reasonably polluted site of the Krishnapatnam sea shore of Nellore coast as portrayed by the writing, and the research center presentation trial of very high Hg levels, found uniquely in extraordinary cases in nature. Further studies ought to be led so as to more readily describe MTs in marine fish tissues and their reactions to substantial metal tainting.

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