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PROTECTIVE BEHAVIOR OF $CACO_3$ AND HUMIC ACIDS FOR BIOACCUMULATION OF HEAVY METALS IN GRASS FISH

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Abstract

This study aims to determine the levels of selected heavy metals (HMs) such as Cd, Pb, Cr, Zn, Cu and Ni in presence of soft/fresh water and at different concentrations of humic acid (HA) and $CaCO_3$, in the liver, gills, skins and flesh of *Grass fish* species. These metals were chosen because of their high level of contamination and toxicity to the fish and human beings as they depend on such fish as food. Large differences in HM concentrations were observed in soft/ fresh water and in different concentrations of HA+$CaCO_3$, between different tissues within the same fish. Different concentrations of HMs were recorded in different organs, but in presence of soft/fresh water liver, gills, skin, and muscle accumulate maximum concentration of each selected metal (Cd, Pb, Cr, Zn, Cu and Ni, respectively). With the addition of different amounts of $CaCO_3$, and HA decreases occurred in the accumulation of selected HMs including Cd, Pb, Cr, Zn, Cu and Ni from 17.37±1.05, 12.57±1.25, 22.24±1.29, 18.61±1.64, 15.87±0.81,and 22.04±2.34 , respectively to 4.37±.66, 4.65 ±0.86, 5.79 ±0.86, 3.54 ±0.69, 9.57 ±0.49, and 6.83 ±0.81 mg kg^{-1}, respectively. The concentrations of HMs were decreased up to four times as compared to soft water with the increases of $CaCO_3$, in hardness. As the concentration of HA reached to the maximum selected level (100 g), double decreases occurred in the accumulation of HMs as compared to $CaCO_3$ treatments. Thus the decreases in HM concentrations ((Cd, Pb, Cr, Zn, Cu and Ni) occurred from 16.10±0.87, 12.42 ±1.96, 20.37±0.54, 17.16 ±2.57, 12.39 ±0.53,and 20.62 ±0.43, respectively to 2.63 ±0.70, 3.35 ±0.45, 3.46 ±0.49, 2.64 ±1.05, 4.43 ±0.57 and 4.33 ±0.48 mg kg^{-1}, respectively. In the presence of HA , the accumulation of HMs were decreased by six times as compared to $CaCO_3$. Furthermore, seven to eight times decreases occurred in the accumulation of selected HMs in fish due to application of both $CaCO_3$ and HA. The lowest concentration was recorded in the fish muscle, while the highest concentration observed in liver samples. Cr and Ni were accumulated more than all other metals. In presence of different concentrations of HA and $CaCO_3$ the accumulation of each metal was decreased. Thus it is observed the treatments of $CaCO_3$ and HA significantly decreased the accumulation of HMs and subsequently their toxicity on fish. However, field/farm level experiments are needed to confirm the long term effects of these materials not on HMs accumulation but also on the health and biomass of fish.

Keywords: heavy metals; humic acid; calcium carbonate; accumulation; fish

Introduction

Water is an important natural resource present in biosphere, it is an essential component of all living organism ranging from micro to macro-organisms. Seas and ocean contribute about 97% of the total water present on the earth surface, while fresh water is about 3% of the whole hydrosphere. The fresh water on the earth surface is very limited (Javed and Usmani 2019)[1]. Life of all organisms is impossible without a proper amount of water in them. So, water is not only a basic block of an ecosystem but also a soul of all organisms in an ecosystem. Without an adequate amount of water life is impossible on the globe. Due to the rapid increase in population, industrialization, urbanization, in many parts of the world water is getting polluted in most places of the earth. The contamination of the fresh water resources with a large amount of pollutant has become a matter of great concern. The natural aquatic ecosystem had been continuously contaminated with toxic HMs release from different natural as well as anthropogenic activities (Javed and Usmani 2011).[2] HMs are classified as, essential and non-essential. Some essential metals are Fe, Cu, Zn, Co, Mn, Ni, which are necessary for various physiological and biochemical functions, on other hand essential HMs have a biological importance, and toxic effect occurs when their metabolic deficiencies occur or at high concentrations. The deficiencies of essential HMs can therefore cause an adverse effect on health, whereas high concentration of essential HMs can also result a negative effect which are equivalent to or worse than those caused by non-essential metals and they can also cause oxidative stress by formation of free radicals (Sivaperumal et al., 2007)[3]. Non-essential HMs are, Cd, Pb, Hg, Cr, which are not needed by the body, are non- biodegradable, and cannot be removed biologically. Toxic HMs also replaced essential metals from the enzymes and cause disrupting of their function. So, HMs make the ecosystem unfavorable for the plants and animals (Henry et al., 2000;[4] Babo.O. F. et al., 2021;[5] Khan et al., 2008)[6]. HMs contaminations have a great effect on the aquatic flora and panna. Among aquatic organism fishes are at the end of the food chain, which cannot be escape from the hazardous effect of the HMs. HMs can enter into the fish body by different rout such as directly from the surrounding water by ingestion of food through alimentary canal, or indirectly from the surrounding via gills and skin. Fish accumulate toxic HMs pollutant in their fatty tissues like liver. (Olaifa et al., 2004;[7] Dobaradaran et al., 2010:[8] Mohammad et al., 2015).[9] Accumulation of HMs depends upon their intake, storage and elimination from the body. So those metals which have high uptake and low elimination from the body tissues of fish are expected to be accumulated to higher levels (Kalay and Canli 2000: [10]Idriss and Ahmad 2015)[11]. HMs are toxic to organisms because they tend to accumulate in body tissues. Compounds of organic pollutants and inorganic HMs accumulate in living things any time when they are taken up and stored faster than they are broken down or extracted or metabolized (Lenntech 2005a)[12]. Inorganic or ionic form of metals are very toxic than organic and complex form of compounds. They are very toxic at early developmental stage of the fish (Mohammad et al., 2015).[9] HMs pollution causes a variety of adverse health effects. These exit over than 20 different HMs toxins that have adverse impacts on human health and each metal toxin will produce different behavioral changes, physiological, biochemical and cognitive changes in an exposed species. The degree to which a cell, tissue, or system organ is affected by HMs toxin depends on the toxin its self, the individual's degree of exposure to the toxin and the dose of toxin. The toxicity of HMs is as follows: Hg > Cd > Cu > Zn > Ni > Pb > Cr > Al > Co. This is only an approximate as the vulnerability of species to individual metals varies. Toxicity of the metal also varies according to the environmental conditions that control the chemical speciation of metals (Hassaan et al., 2016)[13]. Aquatic organism like fish may adversely affected HMs toxin in the environment. Toxic HMs is highly persistent in trace amounts, and can potentially cause severe oxidative stress in aquatic organisms. Thus, these pollutants are highly significant in terms of ecotoxicology (Woo et al., 2009).[14] The toxicity of the HMs mostly depends on the chemistry of water, composition of the sediments in the surface. HMs may enter into the aquatic organism through main three routs which are as; Free metals ions or redials that are adsorbed through respiratory surface such as gills are directly diffused into the blood circulatory system. Free metals ions that are adsorbed onto body surface are diffuse passively into the blood stream, and in last metals that are adsorbed onto food particle and particulates matter may be ingested, as well as free ions ingested with water are diffuse into the blood circulation (Hassaan et al., 2016)[13]. Once HMs enter and accumulated by an aquatic organism, they can be transferred through the higher classes of the food chain. Carnivores at the end of the food Chain including humans, obtain most of their metal burden from the aquatic ecosystem by way of their food, especially where fish are present so there exist the potential for considerable bio-magnifications (Ayandiran et al 2009)[15]. Pollutants in aquatic systems, including HMs, stimulate the reactive oxygen species (ROS) that can damage the biological system of fishes and other aquatic organisms. Fish is a part of diet which has a potential public health concern as it can be contaminated with a range of environmentally persistent chemicals, including HMs. The consumption of fish containing elevated levels of metals is a concern because chronic exposure to HMs can cause health problem (Jiwan and Ajay 2011[16]: Soliman et al., 2006)[17].

Material and methods

Fish (*Grass* fish) were collected from a natural pond along river Swat side and transported to lab in a plastic container in water. For the addition of HMs such as Cd, Pb, Cr, Cu, Zn, and Ni, one ppm solution of each metal prepared from their respective salts and added in to each aquarium. The hardness as $CaCO_3$, HA and both $CaCO_3$+HA were added in different concentrations into each aquarium. The control treatment was not added with $CaCO_3$, HA and both $CaCO_3$+HA, except equal addition of selected HMs. The collected fish were first acclimatized for three weeks in the laboratory conditions before starting these experiments. Three fish of the same size and age were kept in each aquarium of 40 L volume water for the convenient adaptation, and the fish were feed with grasses, wheat bran and or miller bran (choker), and rice hull and husk (choker of rice) in alternate day because fishes were herbivores in nature. Each aquarium was aerated with artificial aeration pump (peristaltic pump) to continuously supply and maintain the oxygen level in water. The treatments of hardness as $CaCO_3$ prepared at different concentrations such as 6.025 g, 12 g, 25 g, 50g and 100 g, HA treatments as 6.025 g, 12 g, 25 g, 50g and 100 g, and both $CaCO_3$+HA as 6.025 g, 12 g, 25 g, 50g and 100 g , while the control treatment was without the addition of these materials. These experiments were continued for 30 d for the exposer of *grass fish* added into each aquarium. The water and the selected chemicals (HMs, HA and $CaCO_3$) were changed twice in a week time. The stock solutions of all metals (Cd, Pb, Cr, Cu, Zn, and Ni), (1000 mg/L) were prepared by dissolving analytical-grade of these chemicals (Merck Chemicals, Darmstadt, Germany) in distilled water.

Sample preparation

Fish samples were cleaned with distilled water and then dissected to get different types of samples. In the laboratory, the total length was taken by scale, while biomass of fish was measured using a digital balance. Tissues of fish were separated into liver, gills, skin and muscle/flesh and each organ wet-weight was also quantified through a digital balance. One gram wet weight of each part was taken in glass beaker and 5 ml of HNO_3 (65%), and 2 ml of $HClO_4$ (70%) were added. Then the mixture was kept on hot plate up to 80 C for 30 min. After cooling, the mixture was filtered through Whatman NO. 40 filter paper and then the volume was made 50 ml with DDW. These extracted samples were analyzed using Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-MES, Perkin Elmer Optima 2100DV, Germany).

Result and Discussion

Protective behaviors of $CaCO_3$ on bioaccumulation of HMs in *grass fish species*

Figure. 1 shows the protective behaviors of $CaCO_3$ on bioaccumulation of selected HMs such as Cd, Pb, Cr, Zn, Cu, and Ni in liver, gills, skins and muscles of *grass fish.* According to the **Fig. 1A**, the accumulation of Cd, Pb, Cr, Cu, Zn, and Ni in liver of *grasss fish species* was higher for soft water as compared to hard water. Similarly, the bioaccumulation of HMs decreased with increasing the concentrations of hardness in water (from a concentration of 6.025 to 12, 25, 50, and 100 g). The highest concentration was observed for Cd, as bioaccumulated in the liver sample of fish in soft water and recorded up to 17.4±1.05 mg kg^{-1} w wt, while the lowest concentration of Cd was accumulated from hard water at maximum concentration of $CaCO_3$ (100 g) and observed as 4.37±0.66 mg kg^{-1} w wt. According to Rathor and Khangarot (2003) [18]: and Pourkhabbaz (2011)[19]), the HMs are more soluble in soft water than the hard water. It means that in soft water the Cd was more available for accumulation in fish as compared to hard water, therefore, it can show toxicity on aquatic animals including fish. In order to compare the effects of hard water at five different concentrations, the maximum amount of Cd was accumulated in liver because of $CaCO_3$ application at the level of 6.025 g. In this treatment, the Cd in liver was accumulated up to 16.5±1.20 mg kg^{-1} w wt basis. However, the lowest amount of Cd was found in the presence of 100 g of $CaCO_3$ which was 4.37±0.66 mg kg^{-1} w wt. The order of accumulation of Cd in different concentrations of $CaCO_3$ were decreased with increasing the of concentration of $CaCO_3$ and Cd was recorded as 17.37±1.05 > 16.5±1.20 > 10.65±0.48 > 7.94 ±0. 81 > 7.64 ±1.15 > 4.37±.66 mg kg^{-1} wet wt.

In case of Pb, the highest concentration was accumulated in liver from soft water which was 12.5±1.25 mg kg^{-1} wet wt (**Fig. 1**). In hard water the highest accumulation of Pb was observed in presence of lowest concentration of $CaCO_3$ (6.025 g), which was 12.46 ±0.58 mg kg^{-1} wet wt, while lowest accumulation was observed in presence of highest concentration of CaCO3 (100g) and was 4.65±0.86 mg kg^{-1} wet wt. In soft water the accumulation was highest because in soft water salt of Pb was more soluble and available for attack on any organs, while in hard water the accumulation was very low because there is and other ions of Ca^{++} present for competition which reduce the attack of metals ions. The order of accumulation of Pb in different concentrations of $CaCO_3$ were decreases as the concentration of $CaCO_3$ from (6.025 to 100 g) increases gradually and were recorded as 12.46 ±0.58 > 9.83±0.05 > 8.39±0.45 > 6.89±0.86 > 4.65±0.86 mg kg^{-1} wet wt. The F and P values are 32.83638 and 1.34E-06, respectively. The F and P values are significant respectively as 91.747024 and 3.89281E-09.

The accumulation of Zn was more in soft water which was 18.61± 1.64 mg kg^{-1} wet wt. The highest concentration of Zn in presence lower concentration of $CaCO_3$ 6.025 g is 13.56±3.42 mg kg^{-1} wet wt, while lowest accumulation in presence of 100 g $CaCO_3$ was 3.54±0.69 mg kg^{-1} wet wt. In varying concentrations of $CaCO_3$ the order of accumulation of Zn from hard water to very hard water were 13.56±3.42 > 13.24±0.52 > 9.21±0.94 > 6.95±1.39 > 3.54±0.69 mg kg^{-1} wet wt, their F and P values are respectively significant as 19.243333 and 2.34706E-05.

The accumulation of Cu in presence of soft water was highest and observed as 15.87± 0.81 mg kg^{-1} w wt. In presence of lowest concentration of heard water (6.025 g), the highest accumulation was observed for Cu (15.87±0.81 mg kg^{-1} w wt). In presence of highest concentration of CaO_3 (100 g), Cu accumulation was noticed less than all other selected concentrations such as 9.57 ±0.49 mg kg^{-1} w wt. The order of Cu accumulation in the presence of varying concentrations of hard water (6.025, 12, 25, 50 and 100 g) were 15.87±0.81, 19.61±0.45, 12.69±0.04, 9.13±1.05, 9.57 ±0.49 mg kg^{-1}, respectively on w wt basis. The values of F and P were significant and found as 5.91±0.81 and 103.334120, respectively.

Accumulation of Ni in presence of soft water is 22.04±2.34 mg kg^{-1} w wt, as in soft water salts are more available than hard water because there was no other competitor present to stop the attack of salt ions. In presence of lowest concentration of $CaCO_3$ Ni accumulate in higher concentration which is 21.36±0.600 mg kg^{-1} w wt, while in presence of highest concentration of $CaCO_3$ Ni accumulate in small amount which was 6.83±0.81 mg kg^{-1} w wt. The order of accumulation of Ni in varying concentration of $CaCO_3$ are 21.36±0.600>16.68±0.72>14.88±1.75>10.24±1.96>6.83±0.81 mg kg^{-1} w wt, their F and P values are respectively 31.160792 and 1.78364E-06. The order of accumulation wss decreasing as the concentration of the $CaCO_3$ increases. Bioaccumulation of HMs in liver might be as a result of the fact that liver is the only organ which accumulate maximum amount of metal and also play an important role in detoxification of HMs. Liver also consists on a protein known as metallothioneine, which has more binding capacity of metals (Akan et al., 2012).[20] A study conducted by Perschbacher and Wurts (1999)[21] as water hardness increases by calcium ions in catfish (Ictalurus punctatus) its survival increases so many times and the concentration of HMs decreases in different tissues of the body.

The highest accumulation of Cr was observed in soft water by the liver and was 22.24±1.29 mg kg^{-1} wet wt, while lowest concentration was observed in very hard water and was 5.79±0.86 mg kg^{-1} wet wt, and more among all the metals. Highest accumulation of Cr in presence of varying concentration of $CaCO_3$ (6.025, 12, 25, 50, and 100 g) in presence of 6.025 g $CaCO3_3$ which is 20.78±0.71, and lowest accumulation in presence of 100 g $CaCO_3$ is 5.79±0.86 mg kg^{-1} wet wt. The order of accumulation Cr from hard water (varying concentration of $CaCO_{3,}$ from 6.025 to 100 gm) were as 20.78±0.71 > 16.46±1.28> 10.94±0.95> 7.94 ±0.77> 5.79±0.86 mg kg^{-1} wet wt.

According to the **Fig. 1B**, the accumulation of Cd, Pb, Cr, Cu, Zn, and Ni in gills of *grasss fish species* was highest in soft water as compared to hard water. Similarly, the bioaccumulation of HMs decreased with increasing the concentrations of hardness in water (from a concentration of 6.025 to 12, 25, 50, and 100 g). The highest concentration of Cd was observed in soft water in gills sample of fish which is 16.10 ±0.87 mg kg^{-1} w wt, while the lowest concentration of Cd was accumulated in hard water at maximum concentration of $CaCO_3$ (100 g) and was 2.63 ±0.70 mg kg^{-1} w wt. in presence of varying concentration of hard water, the highest accumulation was observed in presence of 6/025 g $CaCO_3$ which is 15.3 ±0.90 mg kg^{-1} w wt, while lowest accumulation was observed in presence of 100 g $CaCO_3$ and is 2.63 ±0.70 mg kg^{-1} w wt. The order of bioaccumulation of Cd in presence of varying concentrations of hard water (6.025, 12, 25, 50, and 100 g) are 15.3 ±0.90,>8.61 ±1.29>7.94 ±1.58>5.65 ±0.81>2.63 ±0.70 mg kg^{-1} w wt, the F and P values are significant respectively 49.2072698 and 1.39551E-07.

The bioaccumulation of Pb in soft water was 12.42 ±1.96 mg kg^{-1} w wt, while more lowest is in very hard water which was 3.35 ±0.45 mg kg^{-1} w wt. In very hard water (100) g $CaCO_{3),}$ the accumulation was lowest which was 3.35 ±0.45 mg kg^{-1} w wt , while in water with a concentration of 6.025 g $CaCO_3$ accumulation was 10.43 ±1.24 mg kg^{-1} w wt. In a varying concentration of $CaCO_3$ Pb accumulate varyingly in a decreasing order which are 10.43±1.24 >8.99±0.10>7.40±0.45>4.61±0.64>3.35 ±0.45 their F and p values are 22.90275 and 9.4E-06

The bioaccumulation of Cr in presence of soft water is 20.37±0.54 mg kg^{-1} w wt. Highest bioaccumulation in presence of very hard water 100 g $CaCO_3$ is 3.46±0.49 mg kg^{-1} w wt, while in presence of 6.025 g $CaCO_3$ was 19.58±0.45 mg kg^{-1} w wt. In presence of low to highest hard water the order of accumulation decreases from higher to lower, 19.58±0.45>15.13±1.73>8.68 ±0.91>5.94±0.77>3.46±0.49 mg kg^{-1} w wt, their F and P values are significant respectively 118.85994 and 8.59099E-10.

The accumulation of Zn in gills from soft water was 17.16±2.57 mg kg^{-1} w wt. In presence of low hard water accumulation was higher and observed as 14.76±0.94 mg kg^{-1} w wt, while in presence of very hard water the accumulation was very small i.e. 2.64±1.05 mg kg^{-1} w wt. The order of bioaccumulation of Zn in presence of varying concentrations of hard water were 14.76 ±0.94>11.84±0.76>6.98±0.81>5.76±0.89>2.64±1.05. Their F and P values were significant and observed as 35.581409 and 8.58623E-07 respectively.

The accumulation of Cu in gills from soft water was 12.39±0.53 mg kg^{-1} w wt. In presence of low hard water 6.025 g $CaCO_3$, accumulation was higher which is 16.24±1.23 mg kg^{-1} w wt, while in presence of very hard water 100 g $CaCO_{3,}$ the accumulation is very small which was 4.43±0.57 mg kg^{-1} w wt. The order of bioaccumulation of Zn in presence of varying concentrations of hard water from (6.025 g to 100 g) are 16.24±1.23>9.61±1.20>7.27 ±0.41>8.20±0.39>4.43±0.57. Their F and P values are respectively significant and are 52.490190 and 9.67489E-08.

The bioaccumulation of Ni in soft water was 20.62±0.43 mg kg^{-1} w wt. In presence of low hard water 6.025 g $CaCO_3$, accumulation was highest which was 18.16±1.25 mg kg^{-1} w wt, while in water with a concentration of 100 g $CaCO_3$ accumulation is 4.33±0.48 mg kg^{-1} w wt. In a varying concentrations of $CaCO_3$ Ni accumulate varyingly in a decreasing order which are 18.16±1.25>16.21±1.77>13.80±0.94>8.20±1.24>4.33±0.48, their F and p values are 60.642265and 4.24711E-08. The bioaccumulation of the HMs in the gills of fish might be a fact that the gills of fish tend to accumulate significantly large amount of HMs comparatively than other organs. The reasons for the higher bioaccumulation of HMs in the gills tissue are, that gills surface is the first target of the water borne HMs. The microenvironment of gills surface consists of an epithelial membrane which consist of phospholipids covered by the mucus layer (Shovon et al., 2017).[22] According to Akan et al, 2012)[20] the gill surface is negatively charged and provides a potential site for gill metals interaction for the positively charged cations. The gills of *grass fish species* tend to bioaccumulate highest concentration of HMs in soft medium in treatment first where the concentration of $CaCO_3$ is zero. The maximum accumulation of HMs in the gills surface is due to the direct contact with water. The large surface areas of the gills increase their metals uptake through facilitated diffusion. In the gill of *grass fish* HMs uptake and accumulation are facilitated by intracellular binding and sequestration. HMs quickly is taken up by the gill of *grass fish species* (Yap et al., 2003). Our result are the same as the finding of the Nehreen (2019)[23] Exposure of fish to six different water hardness led to various accumulation of HMs. HMs are more soluble in soft water than hard waters (Rathore and Khangarot 2003[18]; Pourkhabbaz 2011)[24]. In fresh water as the concentration of calcium carbonate decreases the accumulation of the HMs increases because the competition between HMs and calcium and magnesium ions for the uptake site of gill increases (Javid et al., 2007).[25] As water hardness increases due calcium ions it reduces metals accumulation and toxicity by saturating the gill surface binding sites with Ca^{++} (Pyle et al., 2002;[26] Kiyani et al, 2013).[27]

According to the **Fig. 1. C.** the bioaccumulation of HMs (Cd, Pb, Cr, Zn, Cu, and Ni) in skin of *Grass fish* in soft water and in different concentration of hard water (6.025, 12, 25, 50 and 100 gm $CaCO_3$). Bioaccumulation of Cd in skins of Grass fish species are, in presence of soft water Cd accumulate in highest concentration which was 9.91±1.36 mg kg^{-1} w wt. From hard water highest accumulation of Cd occurs in presence of 6.025 g $CaCO_3$ concentration, which is 10.47 ±1.9 mg kg^{-1} w wt, while lowest accumulation was observed in presence of highest concentration 100 g of $CaCO_3$ which was 1.78±0.63 mg kg^{-1} w wt. The order of bioaccumulation of Cd in varying concentration of hard water are decreasing from highest to lowest which are 10.47±1.9>5.70±0.42>4.84±0.90>3.63±0.74>1.78±0.63. The F and P values are significant and were 21.5399964 and 1.30091E-05.

The bioaccumulation of HMs (Cd, Pb, Cr, Zn, Cu, and Ni) in skin of *Grass fish* in soft water and in different concentration of hard water (6.025, 12, 25, 50 and 100 gm $CaCO_3$). Bioaccumulation of Cd in skins of Grass fish species are, in presence of soft water Cd accumulate in highest concentration which is 9.91±1.36 mg kg^{-1} w wt. From hard water highest accumulation of Cd occurs in presence of 6.025 g $CaCO_3$ concentration, which was 10.47±1.9 mg kg^{-1} w wt, while lowest accumulation was observed in presence of highest concentration 100 g of $CaCO_3$ which is 1.78±0.63 mg kg^{-1} w wt. The order of bioaccumulation of Cd in varying concentration of hard water are decreasing from highest to lowest which are 10.47±1.9>5.70±0.42>4.84±0.90>3.63 ±0.74>1.78±0.63. The F and P values are significant and were 21.5399964 and 1.30091E-05.

Bioaccumulation of Pb in skin of *Grass fish* in soft water is 5.84±0.86 mg kg^{-1} w wt. Bioaccumulation of Pb in skins of Grass fish species, in presence of 6.025 g $CaCO_3$ concentrations, which is 5.83±0.77 mg kg^{-1} w wt, while lowest accumulation was observed in presence of highest concentration 100 g of $CaCO_3$ which was 2.5±0.44 mg kg^{-1} w wt. The order of bioaccumulation of Pb in varying concentration of hard water are decreasing from highest to lowest which are 5.83±0.77>5.16± 0.91>4.68±0.81>3.5±0.44>2.5±0.44. The F and P values are significant and were 6.651712and 0.003463.

Bioaccumulation of Cr in skin of *Grass fish* in soft water was 14.31±2.62 mg kg^{-1} w wt. Bioaccumulation of Cr in skins of Grass fish species, in presence of 6.025 g $CaCO_3$ concentrations, which was 16.20±0.47 mg kg^{-1} w wt, while lowest accumulation was observed in presence of highest concentration 100 g of $CaCO_3$ which was 2.48 ±1.32mg kg^{-1} w wt. The order of bioaccumulation of Cr in varying concentration of hard water are decreasing from highest to lowest which are 16.20±0.47>11.22±1.11>6.98±0.81>4.24±0.53>2.48±1.32.The F and P values are significant and were 33.3633283 and 1.22542E-06.

The accumulation of Zn in skin from soft water was 10.4±1.36 mg kg^{-1} w wt. In presence of low hard water accumulation was higher which was 12.43±1.90 mg kg^{-1} w wt, while in presence of very hard water the accumulation is very small which was 1.57±0.42 mg kg^{-1} w wt. The order of bioaccumulation of Zn in presence of varying concentration of hard water were 12.43±1.90>8.98 ±0.72>5.16±0.49>3.73±0.84>1.57±0.42. Their F and P values are respectively significant and are 31.0432795 and 1.82095E-06.

The accumulation of Cu in skin from soft water was 10.1±1.75 mg kg^{-1} w wt. In presence of low hard water accumulation was higher which was 13.19±0.46 mg kg^{-1} w wt, while in presence of very hard water the accumulation is very small which was 3.7±0.83 mg kg^{-1} w wt. The order of bioaccumulation of Cu in presence of varying concentration of hard water were 13.19±0.46>7.46 ±0.99>6.46±0.99>6.55±0.79>3.7±0.83. Their F and P values are respectively significant and are 19.7846651and 2.0315E-05.

The accumulation of Ni in skin from soft water was 11.43±2.52 mg kg^{-1} w wt. In presence of low hard water accumulation was higher which is 10.36±1.24 mg kg^{-1} w wt, while in presence of very hard water the accumulation was very small which was 3.28±0.97 mg kg^{-1} w wt. The order of bioaccumulation of Ni in presence of varying concentration of hard water were 10.36±1.24>8.69±2.91>8.14±1.65>6.73±0.77>3.28±0.97. Their F and P values are respectively significant and are 19.9884872and 1.92571E-05. In our study the fish accumulate highest amount of HMs in skin from soft water and it is because of the reason that skin adsorb some amount of HMs with direct contact to water and some amount from circulation of blood. However, higher intake of HMs than normal can cause adverse health problem like kidney and liver damage (Uysal 2011)[28]. When the concentrations of $CaCO_3$ (Hardness) increases accumulation of HMs decreases in skin, because the solubility of $CaCO_3$ in hard water decrease and availability of cations for accumulation. The results of our study are similar to (Kiyani et al, 2013).[27] Our results indicate that as the concentration of Ca^{2+} increases in the water the accumulation of HMs decreases. In contrast, as the water hardness increases, the degree of HMs accumulation in the body tended to decrease (Choi et al., 2022).[29]

According to **Fig. 1D**, the level of concentrations of HMs in muscle of *Grass species* of fish in soft water where concentration of $CaCO_3$ is 0 g, and in different water hardness where concentrations of $CaCO_3$ range from 6.025, 12, 25, 50, and 100 g. In soft water Cd accumulate more in soft water than the hard water which was 7.36±1.72 mg kg^{-1} w wt. In presence of hard water highest accumulation was achieved in presence of 6.025 g $CaCO_3$, which was 5.46±1.23 mg kg^{-1} w wt, while lowest was in presence of 100 g $CaCO_3$ which was 1.23±0.61 mg kg^{-1} w wt. The order of bioaccumulation of Cd from hard water to very hard water in muscle were 5.46±1.23>4.73±0.78>3.70±0.94>3.21±1.67>1.23±0.61. The F and P values are as 5.32077415 and 0.008319968. The bioaccumulation of Cd decreases four times from lower hard water to very hard water.

The level of concentrations of Pb in muscle of *Grass species* of fish in soft water where concentration of $CaCO_3$ was 0 g, and in different water hardness where concentrations of $CaCO_3$ range from 6.025, 12, 25, 50, and 100 g. In soft water Pb accumulate more in soft water than the hard water which was 4.09±0.47mg kg^{-1} w wt. In presence of low hard water highest accumulation was achieved in presence of 6.025 g $CaCO_3$, which is 3.24±0.44mg kg^{-1} w wt, while lowest was in presence of 100 g $CaCO_3$ which was 0.87±0.08 mg kg^{-1} w wt. The order of bioaccumulation of Cd from low hard water to very hard water in muscle were 3.24±0.44> 3.02±0.44>2.61±0.90>2.36±0.40>0.87±0.08. The F and P values are as 8.580891and 0.001171. The pattern of accumulation of Pb decreases seven times from lower concentration of hard water to highest concentration.

Level of accumulations of Cr in muscle of *Grass fish species* in soft water where concentrations of $CaCO_3$ is 0 g, and in different water hardness where concentrations of $CaCO_3$ range from 6.025, 12, 25, 50, and 100 g. In soft water Cr accumulate 8.61±1.23 mg kg^{-1} w wt. In presence of hard water highest accumulation was achieved in presence of 6.025 g $CaCO_3$, which was 8.64±0.47 mg kg^{-1} w wt, while lowest was in presence of 100 g $CaCO_3$ which was 1.53±0.47 mg kg^{-1} w wt. The order of bioaccumulation of Cr from hard water to very hard water in muscle were 8.64±0.47>8.89±10.81>4.21±0.47>2.27±0.28>1.53±0.47. The F and P values are as 45.2831395and 2.23142E-07.

Level of accumulations of Cr in muscle of *Grass fish species* in soft water where concentrations of $CaCO_3$ is 0 g, and in different water hardness where concentrations of $CaCO_3$ range from 6.025, 12, 25, 50, and 100 g. In soft water Cr accumulate 8.61±1.23 mg kg^{-1} w wt. In presence of hard water highest accumulation was achieved in presence of 6.025 g $CaCO_3$, which is 8.64±0.47 mg kg^{-1} w wt, while lowest was in presence of 100 g $CaCO_3$ which is 1.53±0.47 mg kg^{-1} w wt. The order of bioaccumulation of Cr from hard water to very hard water in muscle are 8.64±0.47>8.89±10.81>4.21±0.47>2.27±0.28>1.53±0.47. The F and P values are as 45.2831395and 2.23142E-07.

Level of accumulations of Zn in muscle of *Grass fish species* in soft water where concentrations of $CaCO_3$ is 0 g, and in different water hardness where concentrations of $CaCO_3$ range from 6.025, 12, 25, 50, and 100 g. In soft water Zn accumulate 8.64±0.46 mg kg^{-1} w wt. In presence of hard water highest accumulation was achieved in presence of 6.025 g $CaCO_3$, which was 8.27± 0.41 mg kg^{-1} w wt, while lowest was in presence of 100 g $CaCO_3$ which was 1.04±0.04 mg kg^{-1} w wt. The order of bioaccumulation of Cu from hard water to very hard water in muscle were 8.27±0.41>6.61±0.45>4.5±0.81>2.46±0.49>1.04±0.04. The F and P values are as 77.0817000 and 1.06859E-08.

Level of accumulations of Ni in muscle of *Grass fish species* in soft water where concentrations of $CaCO_3$ is 0 g, and in different water hardness where concentrations of $CaCO_3$ range from 6.025, 12, 25, 50, and 100 g. In soft water Cr accumulate 7.98±1.33 mg kg^{-1} w wt. In presence of hard water highest accumulation was achieved in presence of 6.025 g $CaCO_3$, which was 8.54±1.22 mg kg^{-1} w wt, while lowest was in presence of 100 g $CaCO_3$ which was 1.28±0.41mg kg^{-1} w wt. The order of bioaccumulation of Ni from hard water to very hard water in muscle were 8.54±1.22>6.46±0.49>5.24±0.44>5.24±0.44>1.28±0.41. The F and P values are as 19.9884872 and 1.92571E-05. As these finding are similar to the results of our study, we inferred that in higher water hardness, the Ca^{2+} ions present in large amounts in water not only decreased the solubility of HMs but also decreased its degree of accumulation, as HMs ions are cations and are absorb via the same pathway as Ca^{2+} ions through the gills. Our result and the finding of the (Choi et al., 2022;[29] Alsop et al., 2011)[30] are the similar as the concentration of HMs dissolved in the water decreased as the hardness of water increased.

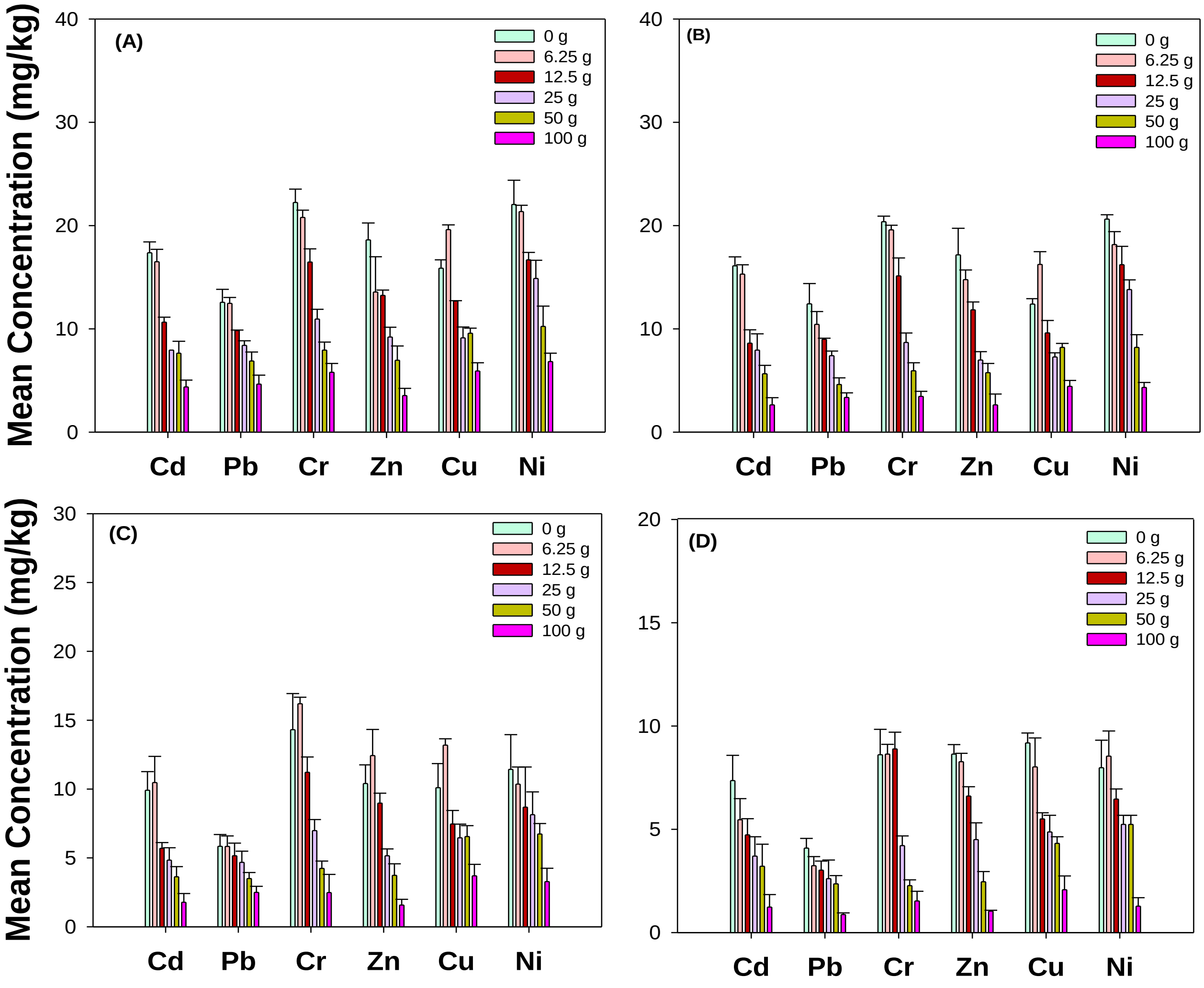

Fig. 1. Bioaccumulation of selected heavy metals in A) liver B) gills C) skin and D) muscles of *grass fish species* in different concentrations of CaCO3

Effects of HA on bioaccumulation of Cd, Pb, Cr, Zn, Cu, and Ni in liver, gills, skin and muscles of *Grass fish species*.

According to the findings **Figure 2, (a) Liver. (b) Gill (c) Skins (d) muscle** were investigate the effects of HA on bioaccumulation of Cd, Pb, Cr, Zn, Cu, and Ni in liver, gills, skin and muscles of grass fish species. According to **Fig. 2. a**. bioaccumulation of HMs (Cd, Pb, Cr, Zn, Cu, and Ni) in liver, gills, skin and muscles, in fresh water (0 gm HA, and in presence of different concentrations of HA (rang from 6.025, 12, 25, 50, and 100 g HA).

In presence of soft water liver accumulate maximum amount of Cd which was 15 77±1.96 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cd was accumulated in presence of 6.025 g HA which was 14.5±0.52 mg kg^{-1} w wt, while minimum concentration of Cd was accumulated in presence of 100 g HA which was 4.28±1.23 mg kg^{-1} w wt. The accumulation of Cd from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 14.5±0.52>11.14±1.10>10.10 ±1.49>7.94±0.86>4.28±1.23.Their F and P values are significant respectively, which are 21.73155743 and 1.24155E-05.

In presence of soft water liver accumulate maximum amount of Pb which was 13.17±1.25 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Pb was accumulated in presence of 6.025 g HA which was 10.82±1.25 mg kg^{-1} w wt, while minimum concentration of Pb was accumulated in presence of 100 g HA which was 4.09±0.86 mg kg^{-1} w wt.

The accumulation of Pb from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 10.82 ±1.25>10.25±1.28>7.53±1.30>6.91±0.77>4.09±0.86.Their F and P values are significant respectively, which are 16.03551and 5.98E-05

In presence of soft water liver accumulation of Cr was 23.63±1.14 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cr was accumulated in presence of 6.025 g HA which was 24.16±0.41mg kg^{-1} w wt, while minimum concentration of Cr was accumulated in presence of 100 g HA which was 4.5±0.65mg kg^{-1} w wt. The accumulation of Cr from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 24.16±0.41>19.19±0.77>15.04 ±6.06>8.76±0.77>4.5±0.65.Their F and P values are significant respectively, which are 2.016838467 and 0.148235591.

In presence of soft water liver accumulate maximum amount of Zn which was 15.13±2.09 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Zn was accumulated in presence of 6.025 g HA which was 11.01±1.38 mg kg^{-1} w wt, while minimum concentration of Zn was accumulated in presence of 100 g HA which was 5.09±0.39mg kg^{-1} w wt. The accumulation of Zn from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 14.47±1.07>11.01±1.38>9.61±0.44>7.42±1.19>5.09±0.39.Their F and P values are significant respectively, which are 19.97913677 and 1.93042E-05.

In presence of soft water liver accumulate maximum amount of Cu which was 20.16±0.49 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cu was accumulated in presence of 6.025 g HA which was 19.61±0.45 mg kg^{-1} w wt, while minimum concentration of Cu was accumulated in presence of 100 g HA which was 5.84±0.13 mg kg^{-1} w wt. The accumulation of Cu from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 19.61±0.45>15.19±1.67>12.55±0.41>8.21±0.40>5.84±0.13.Their F and P values are significant respectively, which are 20.56082468and 1.66132E-05.

In presence of soft water liver accumulate maximum amount of Ni which was 20.94±0.77 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Ni was accumulated in presence of 6.025 g HA which was 16.15±0.5 mg kg^{-1} w wt, while minimum concentration of Ni was accumulated in presence of 100 g HA which was 6.79±0.86 mg kg^{-1} w wt. The accumulation of Ni from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 16.15±0.5>14.58±0.41>11.31±1.24>9.22±0.94>6.79±0.86.Their F and P values are significant respectively, which were 70.88468946 and 1.73328E-08. Because of the increase in concentration of HA the accumulation of Cd decreases up to four times. Accumulation of HMs is in fresh water is highest. The liver is reported to be the only organ for bioaccumulation and thus, has been extensively used to reduce the toxic effects of xenobiotics. Once the toxic substances like HMs cross the biological barriers and enter the bloodstream, they will reach and accumulate in the internal organs of fish, the liver is likely to be the best choice (Begum 2004;[31] Majnoni et al., 2014;[32] Yancheva et al., 2015)[33]. According to Mohamed, 2009[35] due to its large blood supply liver is a target organ which causes noticeable toxicant exposure. According to Yancheva et al., 2015.[33], it is an organ of detoxification and essential for both metabolism and for excretion of toxic substances in the body. Many studies showed that different toxicant accumulates in metabolic organs such as in liver and kidneys (Karadade et al., 2004;[37] Rilbeiro et al., 2005)[36]. The results of control in our study are similar to Amani 2012.[37], exposed *Channa punctatus* to three different concentration of zinc sulfate for different exposure time. *Channa punctatus* accumulate lower concentration of HMs in 7 days exposure time which is 9.60 mg kg^{-1} and in 28 days exposure time accumulate 28.04 mg kg^{-1}. It means that in control medium where zero gm of HA are present can accumulate maximum amount of HMs, because there is no competitor present to stop accumulation of HMs from soft water in liver.

According to **Fig. 2. B.** investigate the effects of HA on bioaccumulation of Cd, Pb, Cr, Zn, Cu, and Ni in gills *Grass fish species*. According to **Fig. 2. B**. bioaccumulation of HMs (Cd, Pb, Cr, Zn, Cu, and Ni) in gills from fresh water (0 gm HA, and in presence of different concentrations of HA (rang from 6.025, 12, 25, 50, and 100 g HA).

In presence of soft water gills accumulate maximum amount of Cd which was 15.5±1.20 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cd was accumulated in presence of 6.025 g HA which was 14.28±1.38 mg kg^{-1} w wt, while minimum concentration of Cd was accumulated in presence of 100 g HA which was 2.57±0.95 mg kg^{-1} w wt. The accumulation of Cd from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 14.28±1.38>8.29±0.43>6.42±1.24>5.85±1.31>2.57±0.95.Their F and P values were significant respectively, which were 39.73473204 and 4.64571E-07.

In presence of soft water gills accumulate maximum amount of Pb which was 12.20±0.8 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Pb was accumulated in presence of 6.025 g HA which was 3.5±0.46 mg kg^{-1} w wt, while minimum concentration of Pb was accumulated in presence of 100 g HA which was 4.09±0.86 mg kg^{-1} w wt. The accumulation of Pb from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 8.30±0.78>9.28±0.92>7.24 ±0.44>4.5 ±0.52>3.5±0.46.Their F and P values were significant respectively, which were 42.16067and 3.33E-07.

In presence of soft water gills accumulation of Cr was 22.52±2.54 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cr was accumulated in presence of 6.025 g HA which was 20.64±1.24mg kg^{-1} w wt, while minimum concentration of Cr was accumulated in presence of 100 g HA which was 3.13±0.96 mg kg^{-1} w wt. The accumulation of Cr from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 20.64±1.24>16.79 ±0.94>11.07±1.96>6.53±0.47>3.13±0.96.Their F and P values were significant respectively, which were 52.51412832 and 9.64986E-08.

In presence of soft water gills accumulate maximum amount of Zn which was 15.40±0.28 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Zn was accumulated in presence of 6.025 g HA which was 13.23±0.32 mg kg^{-1} w wt, while minimum concentration of Zn was accumulated in presence of 100 g HA which was 3.98±1.49 mg kg^{-1} w wt. The accumulation of Zn from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 13.23±0.32>11.24±0.52>7.20±0.47>5.20 ±0.55>3.98±1.49.Their F and P values were significant respectively, which were 78.67743342and 9.4919E-09.

In presence of soft water gills accumulate maximum amount of Cu which was 18.61±0.86 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cu was accumulated in presence of 6.025 g HA which was 17.26±0.68 mg kg^{-1} w wt, while minimum concentration of Cu was accumulated in presence of 100 g HA which was 3.00±.005 mg kg^{-1} w wt. The accumulation of Cu from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100g) which were 17.26 ±0.68>13.71±1.23>9.54±0.40>6.77±0.59>3.00±.005.Their F and P values were significant respectively, which are 48.73007929 and 0.000134288.

In presence of soft water gills accumulate maximum amount of Ni which was 18.14±1.20 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Ni was accumulated in presence of 6.025 g HA which was 15.31±0.55 mg kg^{-1} w wt, while minimum concentration of Ni was accumulated in presence of 100 g HA which was 3.80±0.76 mg kg^{-1} w wt. The accumulation of Ni from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 15.31±0.55>13.1±0.41>9.91 ±1.36>7.28±0.41>3.80±0.76.Their F and P values were significant respectively 73.82962083 and 1.37067E-08. With increase in concentrations of HA and exposure time for accumulation of HMs decreases, because HA have an ability to modify the chemical speciation of a HMs and result in decrease of its bioavailability in first step, and in second step HA interfere with the permeability effect of cell membrane and effect the HMs accumulation. In the third step it is certainly will establish that there are many functional groups attached to HA, which are its component and contain suitable atoms, which are capable of acting as a ligand for HMs. Another possibility through which HA decrease the accumulation of HMs may be because of the success of HA in forming complexes with HMs through the removal of proton from carboxyl groups (Mungkung et al., 2001).[38]

According to **Fig. 2.C.** bioaccumulation of HMs in skin, from soft water and from different concentration of HA.

In presence of soft water skin accumulate maximum amount of Cd which was 11.84±0.73 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cd was accumulated in presence of 6.025 g HA which was 7.80±0.82 mg kg^{-1} w wt, while minimum concentration of Cd was accumulated in presence of 100 g HA which was 1.66±0.91 mg kg^{-1} w wt. The accumulation of Cd from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 7.80±0.82>5.8±0.77>5.07 ±1.67>3.2±0.84>1.66±0.91.Their F and P values were significant respectively, 24.48350955 and 6.58781E-06.

In presence of soft water skin accumulate maximum amount of Pb which was 7.84 ±0.86mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Pb was accumulated in presence of 6.025 g HA which was 6.37±0.73 mg kg^{-1} w wt, while minimum concentration of Pb was accumulated in presence of 100 g HA which was 4.09±0.86 mg kg^{-1} w wt. The accumulation of Pb from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 6.37±0.73>7.64±0.47>4.94±0.81>4.5 ±0.44>2.61±0.44.Their F and P values were significant respectively, 18.80979 and 2.64E-05.

In presence of soft water skin accumulation of Cr was 13.89±1.30mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cr was accumulated in presence of 6.025 g HA which was 13.57±1.64 mg kg^{-1} w wt, while minimum concentration of Cr was accumulated in presence of 100 g HA which was 2.27±0.49 mg kg^{-1} w wt. The accumulation of Cr from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 13.57±1.64>11.82±2.00>7.67 ±1.15>4.84±0.78>2.27±0.49.Their F and P values were significant respectively, 26.38758468 and 4.40537E-06.

In presence of soft water skin accumulate maximum amount of Zn which was 10.50±1.71 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Zn was accumulated in presence of 6.025 g HA which was 8.20±0.47 mg kg^{-1} w wt, while minimum concentration of Zn was accumulated in presence of 100 g HA which was 1.76±0.24 mg kg^{-1} w wt. The accumulation of Zn from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 8.20±0.47>6.87±0.81>5.61±1.71>4.09±0.47>1.76±0.24.Their F and P values were significant respectively,16.11300594 and 5.83377E-05.

In presence of soft water skin accumulate maximum amount of Cu which was 10.15±0.77 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cu was accumulated in presence of 6.025 g HA which was 13.19±0.46 mg kg^{-1} w wt, while minimum concentration of Cu was accumulated in presence of 100 g HA which was 2.31±0.39 mg kg^{-1} w wt. The accumulation of Cu from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100g) which were 13.19±0.46>9.50±0.51>7.87±0.09>5.91±0.72>2.31±0.39.Their F and P values were significant respectively, 13.63291033 and 4.84857E-05.

In presence of soft water skin accumulate maximum amount of Ni which was 15.73±1.43 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Ni was accumulated in presence of 6.025 g HA which was 10.95±0.05 mg kg^{-1} w wt, while minimum concentration of Ni was accumulated in presence of 100 g HA which was 2.87±0.60 mg kg^{-1} w wt. The accumulation of Ni from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were

10.95±0.05>11.18±1.25>6.87±0.81>4.88±0.76>2.87±0.60.Their F and P values are significant respectively, 46.93234491and 1.82373E-07.

According to **Fig. 2. D**. the bioaccumulation of HMs in presence of soft water (concentration of HA is 0gm or soft water).

In presence of soft water skin accumulate maximum amount of Cd which was 6.80±0.78 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cd was accumulated in presence of 6.025 g HA which was 5.39±1.74 mg kg^{-1} w wt, while minimum concentration of Cd was accumulated in presence of 100 g HA which was 1.16±0.26 mg kg^{-1} w wt. The accumulation of Cd from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 5.39±1.74>4.76±0.81>3.74±0.52>3.10±0.33>1.16±0.26.Their F and P values are significant respectively, 9.610047324 and 0.000704654.

In presence of soft water skin accumulate maximum amount of Pb which was 5.42±1.27 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Pb was accumulated in presence of 6.025 g HA which was 5.56±0.77mg kg^{-1} w wt, while minimum concentration of Pb was accumulated in presence of 100 g HA which was 1.35±0.44 mg kg^{-1} w wt. The accumulation of Pb from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which are 5.56±0.77>5.98±1.41>3.90±0.05>2.69 ±0.50>1.35±0.44.Their F and P values were significant respectively, 8.67616 and 0.001116.

In presence of soft water skin accumulation of Cr was 10.87±1.41 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cr was accumulated in presence of 6.025 g HA which was 10.94±1.44 mg kg^{-1} w wt, while minimum concentration of Cr was accumulated in presence of 100 g HA which was 0.83±0.12 mg kg^{-1} w wt. The accumulation of Cr from varying concentration of HA were in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 10.94±1.44>8.93±0.42>3.97±0.44>2.62 ±0.56>0.83±0.12.Their F and P values are significant respectively, 49.16869138 and 1.40172E-07.

In presence of soft water skin accumulate maximum amount of Zn which was 7.98±0.81 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Zn was accumulated in presence of 6.025 g HA which was 8.20±0.47 mg kg^{-1} w wt, while minimum concentration of Zn was accumulated in presence of 100 g HA which was 1.11±0.27mg kg^{-1} w wt. The accumulation of Zn from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which are 6.57±0.99>5.87±0.81>4.35±0.76>2.73 ±1.60>1.11±0.27.Their F and P values were significant respectively, 14.0117783 and 0.000117323.

In presence of soft water skin accumulate maximum amount of Cu which was 7.62±1.58 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Cu was accumulated in presence of 6.025 g HA which was 7.11±1.85 mg kg^{-1} w wt, while minimum concentration of Cu was accumulated in presence of 100 g HA which was 1.03±0.05mg kg^{-1} w wt. The accumulation of Cu from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100g) which were 7.11±1.85>5.04±1.35>4.43±0.87>4 ±0.46>1.03±0.05.Their F and P values were significant respectively, 16.71127471 and 4.84857E-05.

In presence of soft water skin accumulate maximum amount of Ni which was 9.24±0.9 mg kg^{-1} w wt. From HA containing water (6.025, 12, 25, 50, and 100 HA) maximum concentration of Ni was accumulated in presence of 6.025 g HA which was 8.92±0.94 mg kg^{-1} w wt, while minimum concentration of Ni was accumulated in presence of 100 g HA which was 1.21±0.32 mg kg^{-1} w wt. The accumulation of Ni from varying concentration of HA are in decreasing order as HA increasing from (6.025, 12, 25, 50, and 100 g) which were 8.92±0.94>0.16±0.49>5.11 ±0.41>4.38±0.51>1.21±0.32.Their F and P values were significant respectively, 48.20738362 and 1.56741E-07. Zubcov et al., (2012)[39] studied accumulation of Zn in skin and muscle in different fish species like *R. rutilus heckeli, A. brama, C. auratus gibelio* and the result of their finding are 66.0 mg kg^{-1}, 64.0 mg kg^{-1} and 55.2 mg kg^{-1} for skin and for muscle result are 28.5 mg kg^{-1}, 26.4 mg kg^{-1} and 30.2 mg kg^{-1}. The muscle is considered as a non-active tissue in accumulating HMs from the environment (Tekin-Özan and Kir 2008;[40] Visnjic-Jeftic et al. 2010).[41] According to Kamunde and MacPhail, (2011)[42] HA consistently reduced the availability and bioaccumulation of Cd in the tissues of fish.

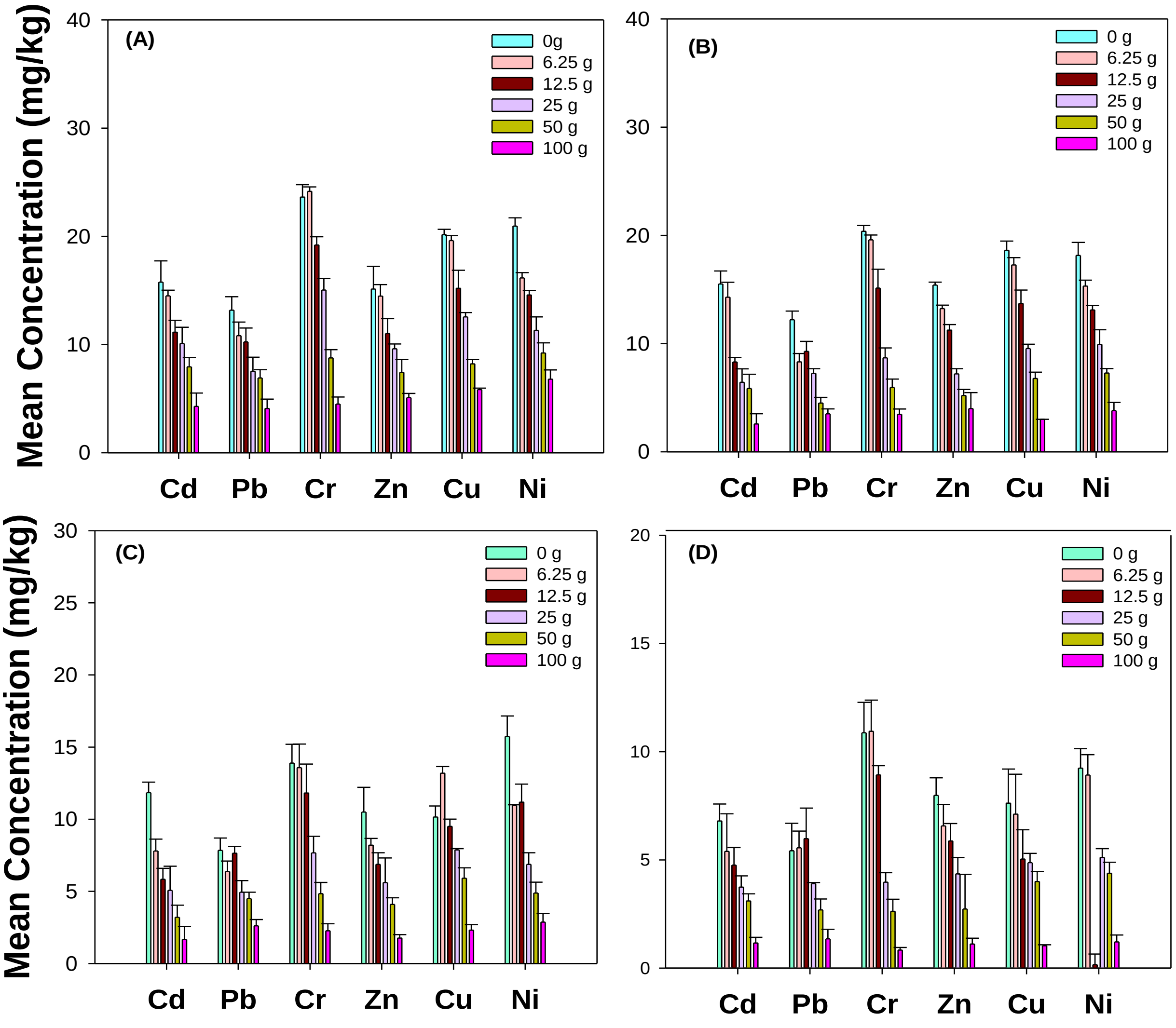

Fig. 2. Bioaccumulation of selected heavy metals in A) liver B) gills C) skin and D) muscles of *grass fish* in different concentrations of Humic acids.

Interactive protective effect of HA + CaCO$_3$ on bioaccumulation of Cd, Pb, Cr, Zn, Cu, Ni, in liver, gills, skin, and muscles of *grass fish species*

According to **Fig. 3.** were investigate the effects of HA+ CaCO$_3$ on bioaccumulation of Cd, Pb, Cr, Zn, Cu, and Ni in liver, gills, skin and muscles of grass fish.

According to **Fig. 3. A.** accumulation of HMs in liver in presence of fresh/ soft water and in different concentrations of both HA+CaCO$_3$. The accumulation of HMs starts from fresh/ soft water as (0 gm HA+CaCO$_3$ in control), and five different concentrations of HA+CaCO$_3$ start from (6.25, 12, 25, 50, and 100 gm) per 40 litter of water. The range of accumulation of Cd in presence of fresh/soft water were 13.98±1.54 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ were 12.21±0.87 and 4.8 ±1.50 mg kg^{-1} w wt. The order of accumulation of Cd were decreasing in varying of concentrations of HA+CaCO$_3$ which were 12.21±0.87>10.90±1.38>7.94±0.77>5.62±3.39>4.8±1.50 mg kg^{-1} w wt. The F and P values were respectively significant 8.4473141and 0.001255327. With the increase in concentration of

HA+CaCO$_3$ the accumulation of Cd decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The range of accumulation of Pb in presence of fresh/soft water was 14.87±1.63 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ are 12.66±0.17 and 5.57±0.49 mg kg^{-1} w wt. The order of accumulation of Pb were decreasing in varying of concentrations of HA+CaCO$_3$ which are 12.66±0.17>9.65±0.87>9.64 ±0.47>7.64±0.94>5.57±0.49 mg kg^{-1} w wt. The F and P values were 27.94161 and 3.23E-06 were significant respectively. With the increase in concentration of HA+CaCO$_3$ the accumulation of Pb decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The range of accumulation of Cr in presence of fresh/soft water was 27.61±2.47 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ are 26.50±2.13 and 4.96±1.00 mg kg^{-1} w wt. The order of accumulation of Cr were decreasing in varying of concentrations of HA+CaCO$_3$ which are 20.20±3.03>20.20±3.03>16.78±1.82>9.95±0.81>4.96±1.00 mg kg^{-1} w wt. The F and P values were significant respectively 38.986833 and 5.16555E-07. With the increase in concentration of HA+CaCO$_3$ the accumulation of Cr decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The range of accumulation of Zn in presence of fresh/soft water was 16.98±0.80 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ are 14.17±1.16 and 5.50±0.45 mg kg^{-1} w wt. The order of accumulation of Zn were decreasing in varying of concentrations of HA+CaCO$_3$ which were 14.17±1.16>7.05±3.30>8.27 ±0.91>6.32±1.24>5.50±0.45mg kg^{-1} w wt. The F and P values were significant respectively 17.13152096 and 4.27191E-05. With the increase in concentration of HA+CaCO$_3$ the accumulation of Zn decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The range of accumulation of Cu in presence of fresh/soft water was 16.54±1.13 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ were 14.2±1.39 and 4.13±2.44 mg kg^{-1} w wt. The order of accumulation of Cu were decreasing in varying of concentrations of HA+CaCO$_3$ which were 14.2±1.39>12.72±0.69>9.87±1.01>9.54±0.54>4.13±2.44 mg kg^{-1} w wt. The F and P values weresignificalt respectively 20.560824 and 1.66132E-05. With the increase in concentration of HA+CaCO$_3$ the accumulation of Cu decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The range of accumulation of Ni in presence of fresh/soft water was 21.58±0.91 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ were 17.07±0.50 and 4.87±0.77mg kg^{-1} w wt. The order of accumulation of Ni are decreasing in varying of concentrations of HA+CaCO$_3$ which were 17.07±0.50>14.58±1.74>11.96±0.40>9.40±0.42>4.87±0.77 mg kg^{-1} w wt. The F and P values were significant respectively 81.595474 and 7.68788E-09. With the increase in concentration of HA+CaCO$_3$ the accumulation of Ni decreases three to four time from higher to lower concentration of HA+CaCO$_3$. Exposure of fishes to highest amount of HMs induced synthesis of metallothioneine protein which is metal binding proteins. Fish have metallothioneine protein in liver, which accumulate, concentrate and detoxify the HMs. In the present study liver of fishes in control treatment accumulate highest amount of HMs, because the gill surface are free from Ca^{2+} ions, which provide a free hand for accumulation and binding of HMs to liver. The liver tissue came second after gill in accumulation of HMs (Akan et al., 2012)[20]. The liver is an organ of accumulation and detoxification of HMs (Yousafzai., 2004).[43] Metals accumulation by aquatic organisms is a two steps process initially it involves a rapid adsorption or binding of the surface, followed by a slower transport into a cell interior. The transport of metals into intracellular space may be either facilitated by either diffusion of the metal across the cell membrane or by active transport by a carrier protein. It is a general acceptation that metals accumulation in tissue of aquatic organism is dependent on exposure concentration and period of time, as well as some other factor like temperature, salinity, and interacting agents (Shukla et al, 2007).[44]

According to **Fig. 3. B.** the bioaccumulation of HMs in gills in presence of soft/fresh water, and in different concentrations of HA+CaCO$_3$. The range of accumulation of Cd in presence of fresh/soft water 12.84±0.83 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ are 11.28±1.28 and 2.25±1.21 mg kg^{-1} w wt. The order of accumulation of Cd are decreasing in varying of concentrations of HA+CaCO$_3$ which are 11.28±1.28>8.64±0.86>6.27±1.68>4 95±0.81>2.25±1.21mg kg^{-1} w wt. The F and P values are 23.55990726 and 8.0894E-06 are significant. With the increase in concentration of HA+CaCO$_3$ the accumulation of Cd decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The bioaccumulation of HMs in gills in presence of soft/fresh water, and in different concentrations of HA+CaCO$_3$. The range of accumulation of Pb in presence of fresh/soft water 13.24±0.97 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ were 12.29±0.38 and 4.20±1.24 mg kg^{-1} w wt. The order of accumulation of Cd were decreasing in varying of concentrations of HA+CaCO$_3$ which were 12.29±0.38>9.03±0.75>6.99±0>5.31±1.24>4.20±1.24 mg kg^{-1} w wt. The F and P values were significant respectively, 34.14362 and 1.07868E-06. With the increase in concentration of HA+CaCO$_3$ the accumulation of Pb decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The bioaccumulation of HMs in gills in presence of soft/fresh water, and in different concentrations of HA+CaCO$_3$. The range of accumulation of Cr in presence of fresh/soft water 25.87 ±2.26 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ are 11.28±1.28 and 2.25±1.21 mg kg^{-1} w wt. The order of accumulation of Cd are decreasing in varying of concentrations of HA+CaCO$_3$ which are 23.84 ±2.15>18.31±2.02>14.58±1.26>6.98±0.81>2.68±0.73 mg kg^{-1} w wt. The F and P values were significant respectively, 60.820183 and 4.1765E-08. With the increase in concentration of HA+CaCO$_3$ the accumulation of Cr decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The bioaccumulation of HMs in gills in presence of soft/fresh water, and in different concentrations of HA+CaCO$_3$. The range of accumulation of Zn in presence of fresh/soft water 15.01±1.67 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ were 12.02±0.77and 2.67±0.92mg kg^{-1} w wt. The order of accumulation of Cd are decreasing in varying of concentrations of HA+CaCO$_3$ which are 12.02±0.77>8.27 ±0.49>6.11±1.36>4.83±0.92>2.67±0.92 mg kg^{-1} w wt. The F and P values are significant respectively, 35.74544888 and 8.37015E-07. With the increase in concentration of HA+CaCO$_3$ the accumulation of Zn decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The bioaccumulation of HMs in gills in presence of soft/fresh water, and in different concentrations of HA+CaCO$_3$. The range of accumulation of Cu in presence of fresh/soft water 14.20±1.30mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ are 11.82±0.42 and 3.32 ±0.41 mg kg^{-1} w wt. The order of accumulation of Cd are decreasing in varying of concentrations of HA+CaCO$_3$ which are 11.82 ±0.42>9.81±0.93>7.87±0.86>5.47±0.56>3.32±0.41 mg kg^{-1} w wt. The F and P values are significant respectively, 48.730079 and 1.47465E-07. With the increase in concentration of HA+CaCO$_3$ the accumulation of Cu decreases three to four time from higher to lower concentration of HA+CaCO$_3$.

The bioaccumulation of HMs in gills in presence of soft/fresh water, and in different concentrations of HA+CaCO$_3$. The range of accumulation of Ni in presence of fresh/soft water 19.588±0.42 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower and highest concentrations of HA+CaCO$_3$ are 15.44±1.02 and 3.51±1.63 mg kg^{-1} w wt. The order of accumulation of Cd were decreasing in varying of concentrations of HA+CaCO$_3$ which are 15.44 ±1.02>12.5±1.23>10.77±1.40>8.27±1.19>3.51±1.63 mg kg^{-1} w wt. The F and P values were significant respectively, 42.398386 and 3.23075E-07. With the increase in concentration of HA+CaCO$_3$ the accumulation of Ni decreases three to four time from higher to lower concentration of HA+CaCO$_3$. In all the tissue of *Grass fish species*, the gills accumulate highest amount of HMs, and it is because of the facts that gills surface is expose to all environmental effect. Gills have a large surface area because of which gills accumulate a highest amount of HMs. (Shukla et al., 2007)[44] reported highest accumulation of Zn in gills followed by liver, and muscle, which are 69.45 mg kg^{-1} wet weight, 24.34 mg kg^{-1} wet weight, and 3.92 mg kg^{-1} wet weight. These result show that when the fishes are exposed to metals in soft water, where no competitor ions are present like Ca^{2+} ion and HA, the tissues of fish can accumulate highest amount of metals. In the present study as the concentration of the HA + $CaCO_3$ were increases the accumulation of Zn were decreases.

According to **Fig. 3.C.** the bioaccumulation of HMs in skins tissues of *Grass fish species* in presence of fresh/soft water and in different concentrations of HA+$CaCO_3$. The bioaccumulation of HMs in skin in presence of soft/fresh water and in different concentrations of HA+$CaCO_3$. The range of accumulation of Cd in presence of fresh/soft water 9.64±0.47 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower to highest concentrations of HA+$CaCO_3$ were 8.35±0.44 and 1.73±0.88 mg kg^{-1} w wt. The order of accumulation of Cd are decreasing in varying of concentrations of HA+$CaCO_3$ which were 8.35±0.44>6.31±1.88>4.46±1.25>4.5 ±0.55>1.73±0.88 mg kg^{-1} w wt. The F and P values were significant respectively, 14.97166532 and 8.44108E-05. With the increase in concentration of HA+$CaCO_3$ the accumulation of Cd decreases three to four time from higher to lower concentration of HA+$CaCO_3$.

The bioaccumulation of HMs in skin in presence of soft/fresh water and in different concentrations of HA+$CaCO_3$. The range of accumulation of Pb in presence of fresh/soft water 7.59±1.15 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower to highest concentrations of HA+$CaCO_3$ were 6.24±0.89and 2.57±1.98 mg kg^{-1} w wt. The order of accumulation of Pb are decreasing in varying of concentrations of HA+$CaCO_3$ which were 6.24 ±0.89>5.98±2>6.98±0.81>3.98±0.81>2.57±1.98 mg kg^{-1} w wt. The F and P values were significant respectively 5.723756 and 0.006297. With the increase in concentration of HA+$CaCO_3$ the accumulation of Pb decreases three to four time from higher to lower concentration of HA+$CaCO_3$.

The bioaccumulation of HMs in skin in presence of soft/fresh water and in different concentrations of HA+$CaCO_3$. The range of accumulation of Cr in presence of fresh/soft water 15.15±2.09 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower to highest concentrations of HA+$CaCO_3$ were 13.94±2.19 and 1.79 ±0.88 mg kg^{-1} w wt. The order of accumulation of Cr were decreasing in varying of concentrations of HA+$CaCO_3$ which are 13.94 ±2.19>8.44±5.35>8.57±1.17>4.80±0.87>1.79±0.88 mg kg^{-1} w wt. The F and P values were significant respectively, 7.764597867 and 0.001810756. With the increase in concentration of HA+$CaCO_3$ the accumulation of Cr decreases three to four time from higher to lower concentration of HA+$CaCO_3$.

The bioaccumulation of HMs in skin in presence of soft/fresh water and in different concentrations of HA+$CaCO_3$. The range of accumulation of Zn in presence of fresh/soft water 13.54±1.28 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower to highest concentrations of HA+$CaCO_3$ are 9.11±0.81 and 1.87±0.92 mg kg^{-1} w wt. The order of accumulation of Zn are decreasing in varying of concentrations of HA+$CaCO_3$ which are 9.11 ±0.81>5.90±1.67>5.90 ±0.86>3.68±0.10>1.87±0.92 mg kg^{-1} w wt. The F and P values were significant respectively, 35.188484 and 9.13083E. With the increase in concentration of HA+$CaCO_3$ the accumulation of Zn decreases three to four time from higher to lower concentration of HA+$CaCO_3$.

The bioaccumulation of HMs in skin in presence of soft/fresh water and in different concentrations of HA+$CaCO_3$. The range of accumulation of Cu in presence of fresh/soft water 10.21±0.47 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower to highest concentrations of HA+$CaCO_3$ were 8.59±1.62 and 3.14±0.28 mg kg^{-1} w wt. The order of accumulation of Cu are decreasing in varying of concentrations of HA+$CaCO_3$ which were 8.59 ±1.62>8.43±1.46>6.73±0.74>5.21±0.40>3.14±0.28 mg kg^{-1} w wt. The F and P values are 13.63291033 and 0.0001342 are significant. With the increase in concentration of HA+$CaCO_3$ the accumulation of Cu decreases three to four time from higher to lower concentration of HA+$CaCO_3$.

The bioaccumulation of HMs in skin in presence of soft/fresh water and in different concentrations of HA+$CaCO_3$. The range of accumulation of Ni in presence of fresh/soft water 16.48±0.43 mg kg^{-1} w wt, while maximum range of accumulation in presence of lower to highest concentrations of HA+$CaCO_3$ were 10.40±1.64 and 2.05±0.51 mg kg^{-1} w wt. The order of accumulation of Ni are decreasing in varying of concentrations of HA+$CaCO_3$ which were 10.40 ±1.64>12.39±0.55>9.06±1.35>6.61±1.29>2.05±0.51mg kg^{-1} w wt. The F and P values were significant respectively, 42.021462 and 3.39668E-07. With the increase in concentration of HA+$CaCO_3$ the accumulation of Ni decreases three to four time from higher to lower concentration of HA+$CaCO_3$.

According to **Fig. 3. D.** the bioaccumulation of different HMs in soft/fresh water, and in different concentration of HA+$CaCO_3$ in muscles tissues of *Grass fish species*. From soft water the accumulation of Cd range 6.92±0.82 mg kg^{-1} w wt, while in hard water from lower concentration of hard water to highest concentration (6.025 g to 100 g), Cd accumulation range from 5.57±0.91 to 2.35±1.56 mg kg^{-1} w wt. The order of accumulation in presence of varying concentration of HA+$CaCO_3$ range from highest to lowest 5.57±0.91>4.57±1.68>3.87±0.44>3.21±0.25>2.35±1.56 mg kg^{-1} w wt. The F and P values were significant respectively, 4.539522481 and 0.014827329.

The bioaccumulation of different HMs in soft/fresh water, and in different concentration of HA+$CaCO_3$ in muscles tissues of *Grass fish species*. From soft water the accumulation of Pb range 6.37±0.85 mg kg^{-1} w wt, while in hard water from lower concentration of hard water to highest concentration (6.025 g to 100 g), Pb accumulation range from 5.31±1.24 to 1.20±0.56 mg kg^{-1} w wt. The order of accumulation in presence of varying concentration of HA+$CaCO_3$ range from highest to lowest 5.31±1.24>4.68±.49>27±1.194>2.98±0.81>1.20±0.56mg kg^{-1} w wt. The F and P values are significant respectively and are 8.050588 and 0.001549.

The bioaccumulation of different HMs in soft/fresh water, and in different concentration of HA+$CaCO_3$ in muscles tissues of *Grass fish species*. From soft water the accumulation of Cr range 8.95±0.81mg kg^{-1} w wt, while in hard water from lower concentration of hard water to highest concentration (6.025 g to 100 g), Cr accumulation range from 10.95±0.81 to 0.81±0.09 mg kg^{-1} w wt. The order of accumulation in presence of varying concentration of HA+$CaCO_3$ range from highest to lowest 10.95±0.81>8.28±1.20>5.39±1.18>33.13±0.44>0.81±0.09 mg kg^{-1} w wt. The F and P values are significant respectively and were 40.07704525 and 4.4283E-07.

the bioaccumulation of different HMs in soft/fresh water, and in different concentration of HA+$CaCO_3$ in muscles tissues of *Grass fish species*. From soft water the accumulation of Zn range 8.51±1.47 mg kg^{-1} w wt, while in hard water from lower concentration of hard water to highest concentration (6.025 g to 100 g), Zn accumulation range from 5.99±0.83 to 1.1±0.16 mg kg^{-1} w wt. The order of accumulation in presence of varying concentration of HA+$CaCO_3$ range from highest to lowest 5.99±0.83>5.90±0.72>4.20 ±0.47>3.37±0.50>1.1±0.16mg kg^{-1} w wt. The F and P values were significant respectively 19.9540279 and 1.94314E-05.

the bioaccumulation of different HMs in soft/fresh water, and in different concentration of HA+$CaCO_3$ in muscles tissues of *Grass fish species*. From soft water the accumulation of Cu range 7.81±.81 mg kg^{-1} w wt, while in hard water from lower concentration of hard water to highest concentration (6.025 g to 100 g), Cu accumulation range from 5.62±0.50 to 1.44±0.48 mg kg^{-1} w wt. The order of accumulation in presence of varying concentration of HA+$CaCO_3$ range from highest to lowest 5.62±0.50>5.5±1.32>4.21±0.47>2.81±0.71>1.44±0.48 mg kg^{-1} w wt. The F and P values are significant respectively, 16.71127471and 4.84857E-05.

The bioaccumulation of different HMs in soft/fresh water, and in different concentration of HA+$CaCO_3$ in muscles tissues of *Grass fish species*. From soft water the accumulation of Ni range 13.73±0.87 mg kg^{-1} w wt, while in hard water from lower concentration of hard water to highest concentration (6.025 g to 100 g) Ni accumulation range from 9.55±1.28 to 1.21±0.23 mg kg^{-1} w wt. The order of accumulation in presence of varying concentration of HA+$CaCO_3$ range from highest to lowest 9.55±1.28>11.83±0.90>8.64±0.47>3.48±0.62>1.21±0.23 mg kg^{-1} w wt. The F and P values were significant respectively, 71.78404542 and 1.61181E-08.

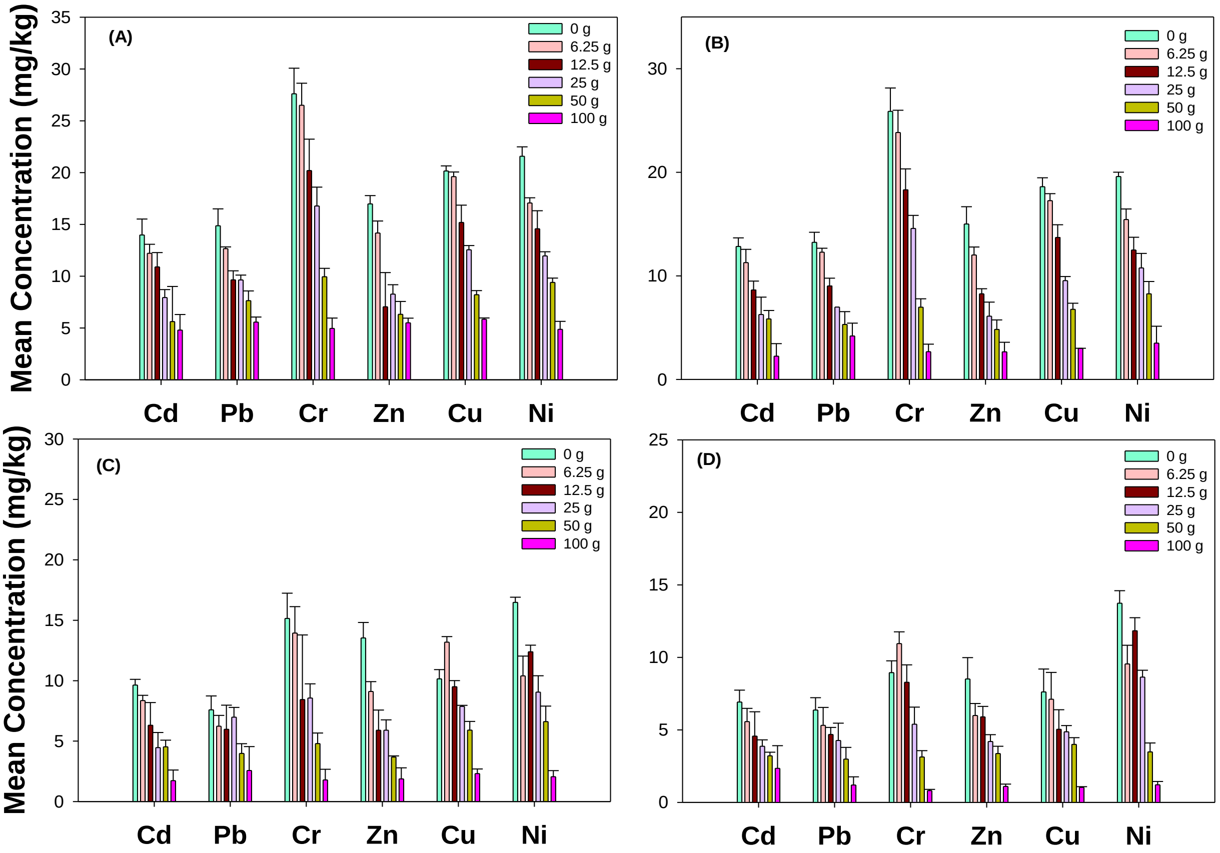

Fig. 3. Bioaccumulation of selected heavy metals in A) liver B) gills C) skin and D) muscles of *grass fish* in different concentrations of CaCO3+Humic acid

Conclusion

Based on the results of this study, the levels of bioaccumulated metals in tissues of *Grass fish species*, the highest levels of all the metals in the present study were observed in liver and gills, while skins and muscles showed lowest value in soft/fresh water. In the presence of different concentration of $CaCO_3$, from lower concentration toward the higher concentration, a successive decrease occurs in accumulation of HMs in different organs. HA are also used as another organic material to check its protective behavior in presence of different HMs in different concentrations. The effects of HA are the same as that of the $CaCO_3$, but its medicinal effect is very beneficial for the growth and protection from the hazardous effect of HMs. It also reduced internal of fish and external infection present in water, and also treat healing of wound. In control medium where there are no material (HA) used for hardness the HMs effect on the behavior of fishes, like reduction occurs on swimming, eating, movement in different sides of the aquarium, they are seen always in rest in the bottom of the aquarium. In the presence of HA the fishes are no more effect. Therefore, HA and $CaCO_3$ decrease the bioaccumulation of HMs.

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