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Sublethal impacts of heavy metals on antioxidant enzymes and biochemical parameters in rohu (*Labeo rohita)*

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Abstract

The present research was designed to study and compare the biochemical parameters in *Labeo rohita* due to exceptional nutritional value of this fish as a protein source in developing countries. The protein and heavy metal (Zn, Cu, Ni) activities were determined spectrophotometrically. The proximate analysis of *L. rohita* collected from different localities of Pakistan revealed that the protein (19.97%) and ash contents (1.76%) were highest in hatchery *L. rohita* while the fat (0.84%), carbohydrate (5.39%) and dry matter contents (24.11%), were maximum in the river *L. rohita*. In comparison to the hatchery and river *L. rohita*, the moisture contents (81.42%) were the highest in farmed fish. During enzymatic analysis, maximum activities of the peroxidase, α-amylase and mutarotase were recorded in hatchery fish whereas the catalase and superoxide dismutase activities were found to be the highest in farmed fish. The maximum accumulation of Cu and Ni metals were observed in hatchery fish and least in farmed fish and the highest Zn accumulation was observed in river fish. Analysis of variance on catalase, peroxidase, α-amylase activity and metal accumulation showed statistically significant differences at $p<0.05$ among sampling sites and fish organs and interaction between sampling sites and fish organs.

Keywords: Aquaculture, Enzymes, Proximate analysis, Antioxidant enzymes, Metals, *Labeo rohita*.

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Introduction

Rapid industrialization is contaminating natural freshwaters by the heavy use of metals mainly iron, zinc, copper, lead, nickel and manganese turning it into a global problem. The concentrations of trace elements are dangerous not only to fish growth and reproduction but also for human beings. Because of increasing industrial practice, heavy metals enter the aquatic environment and are transferred to human beings through the food chain. Minerals perform important roles in osmoregulation, intermediary metabolism and in the formation of skeleton, healthy scales, teeth and bones (Vutukuru *et al*., 2007).

Rohu or roho labeo is a species of the carp family and is a natural inhabitant of freshwater and is present in the rivers of Asian countries (Pakistan, India, Bangladesh, Burma and Nepal). In Pakistan, this species is mostly available in the province of Punjab and due to its non-oily nature; it is widely consumed as food. Fish is an important source of balanced and easily digestible protein, carbohydrates, polyunsaturated fatty acids, minerals i.e. copper, iodine, potassium, phosphorus, iron, and vitamin A and D. Fish proteins have a high biological value and contain all the essential amino acids and are an excellent source of lysine that is limited in vegetable foods (Vladau *et al*., 2008).

Fish tissue, generally the kidney and liver, have antioxidant defense mechanisms consisting of catalase, superoxide dismutase and peroxidase etc. to protect them from the oxidative effect of heavy metals (Basha and Rani, 2003). These enzymes provide the first line of defense against oxygen-derived free radicals and protect the fish tissues from oxidative stress. Antioxidant enzymes can be used as biomarkers of exposure to aquatic contamination (Ahmad *et al*., 2000). However, antioxidant enzymes exhibit different activities between the cells, tissues and organs of saltwater fish and freshwater fish depending upon feeding habitat, environmental conditions and other ecological conditions. On the other hand, catalase and superoxide dismutase enzymes may be highly influenced by a number of conditions viz. temperature, season, salt contents, sex, age and feeding habitats.

Labeo rohita is an emerging fisheries resource and its highly viable significance makes it a promising aquaculture species in Pakistan and other Asian countries. The availability of data regarding antioxidant enzymes and nutritional profile with reference to heavy metal stress on this species is very limited. So, this work was planned to evaluate various biochemical parameters including proteins, lipids, carbohydrates, heavy metals and activity of various enzymes in *L. rohita.*

Materials and methods

The fish, *L. rohita* belongs to the family Cyprinidae of fresh water fishes, were collected from a Fish hatchery at Satiana Road, Faisalabad, Fisheries Research Farms at University of Agriculture, Faisalabad and Taunsa

Barrage Indus River. The nine fish were dissected and the body organs viz. gills, kidney and liver were collected in triplicate for the studies of enzymes and heavy metals.

Proximate analysis of fish meat

The proximate analyses viz. moisture, crude protein, total fat and total ash were assayed as described by Association of Official Analytical Chemists (AOAC, 1990). The moisture percentage was calculated as the loss in weight of meat samples. Total fat and ash was calculated from meat samples. Total fat was calculated by the following formula (AOAC, 1990).

$$\text{Percentage of Total fat} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

Quantitative estimation of protein contents

Determination of protein contents was carried out by Kjeldahl method (AOAC, 1990). In brief, 1 g of dried sample of meat was placed in a digestion flask with 5 g of digestion mixture ($FeSO_4$=5 parts, $CuSO_4$=10 parts and K_2SO_4= 100 parts) and 25-30 mL H_2SO_4. The digestion mixture was boiled for 3 hours on digestion apparatus and the solution was changed into a greenish color. Sample solution (10 mL) and sodium hydroxide (40%) were placed in the digestion apparatus and distilled with steam. Ammonia was collected in 2% boric acid solution (10 mL). When the pink color of 2% boric acid changed to golden color, then ammonia was collected approximately for 2 minutes. Ammonia was titrated against sulphuric acid (0.1 N) where total amount of H_2SO_4 used was noted and the percentage of nitrogen was calculated.

Analysis of antioxidant enzymes

For enzyme analysis, fish was dissected and the organs viz. liver, kidney and gills were collected. Tissue samples to be processed for the determination of enzyme activities were homogenized (1:4, w/v) in phosphate buffer (pH 6.5). Homogenate samples were centrifuged at 10,000 rpm for 15 min. at 4ºC. A supernatant fraction was used to check the activity of various enzymes.

The activity of superoxide dismutase was found by measuring its capacity to inhibit the photoreduction of nitroblue tetrazolium (Worthington, 1988). One unit of superoxide dismutase activity is the amount which is required for 50% reduction in color and was expressed in units of the enzyme (U/mg) and absorbance was noted on a spectrophotometer at 560 nm.

The catalase activity was measured according to the method of Chance and Maehly (1995). Catalase enzyme activity was determined by measuring its ability to reduce the hydrogen peroxide concentration at 240 nm. The reaction mixture (2 mL) contained 1.95 mL of buffered substrate solution and when 0.05 mL enzyme extract was added, the reaction started in the cuvette and absorbance was recorded at 240 nm on the spectrophotometer within 3 minutes.

The activity of peroxidase was determined by measuring its capacity to decrease the hydrogen peroxide concentration at 470 nm (Zia *et al*., 2011). The spectrophotometer was set at 470 nm wavelength, after inserting the blank solution. Then 0.02 mL of crude extract was added in 1 mL of buffered substrate solution and the absorbance was noted after 3 minutes.

α-Amylase is measured by its ability to reduce 3,5-dinitrosalicylic acid (Niaz *et al*., 2010; Mirza *et al*., 2013) and absorbance was noted against a blank solution at 540 nm. Mutarotase is another important enzyme that is responsible for spontaneous mutarotation of sugars and its activity in a supernatant of fish samples was determined according to the method of Mazhar *et al*. (2012).

Determination of heavy metals

In triplicate, fish organs viz. gills, kidney and liver were removed and digested, separately with concentrated nitric acid (HNO_3) and $HClO_4$ by 3:1 ratio. In a 100 ml tube, the sample and 10ml concentrated HNO_3 were added and heated at 100, 150, 200 and 250˚C on a hot plate for 0.5, 0.5, 0.5 and 1.5 hours, respectively. Then $HClO_4$ was added to it. Afterwards we added 2 mL of 1N nitric acid and put it on a hot plate and heated it until the sample was completely digested and became transparent. The digested samples were then transferred to volumetric flasks (50 mL) and the volume was made up by adding deionized water. The samples were filtered through 0.45 µm Millipore membrane filter (Type HV). The filtrate was analyzed for metals viz. Zn, Cu and Ni following the method of Andaleeb *et al*. (2008) by using Atomic Absorption Spectrophotometer Analyst 400-Perkin Elmer (USA).

Statistical analysis

Analysis of variance was performed to find out statistical differences among variables. The results were expressed as mean ± SE. p values<0.05 were regarded as statistically significant.

Results

Proximate analysis of fish meat samples

The proximate analysis of fish meat samples is given in Fig. 1. The maximum protein contents (19.97%) were analyzed in hatchery *L. rohita* while, farmed *L. rohita* exhibited less protein contents (13.76%). The total fat contents were maximum in river (0.84%) and minimum in farmed fish (0.64%).

Lipids are the most important constituents of fish, which determine the fish meat quality. The fat contents of fish are variable from species to species and due to seasonal changes, physiology, feeding, habitats etc. The carbohydrate contents in hatchery fish (1.97%) and in river fish (5.39%) were observed, respectively.

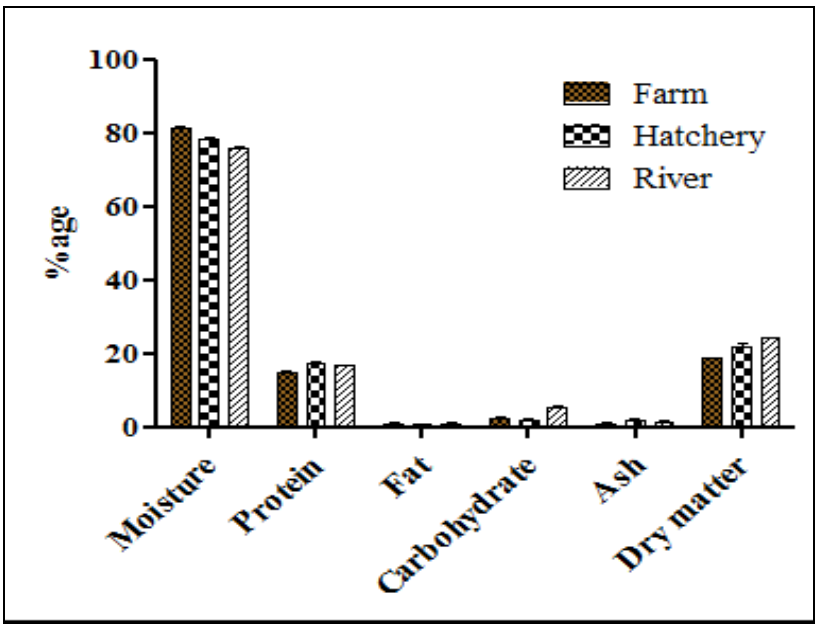

Figure 1: Proximate composition of *Labeo rohita* obtained from different habitats.

Ash and moisture contents were found highest in hatchery and farmed fish, correspondingly. Maximum value of dry matter (24.11%) and its minimum (18.57%) were noted in farmed fish. Body composition of *L. rohita* is influenced by a number of factors i.e. size, weight and feed intake.

Analysis of antioxidant enzymes

Superoxide dismutase activity: Superoxide dismutase (SOD) activity in different organs (liver, kidney, gills) of *L. rohita* is given in Fig. 2. Higher activity of superoxide dismutase was observed in kidney (88.4 U mL^{-1}) of farmed fish while, the least activity was found in liver (74.68 U mL^{-1}) of hatchery fish. The activity of SOD in organs of *L. rohita* from different localities showed that an increase in activity formed the trend farm>river>hatchery fish.

The SOD activity was found lower in polluted sites and higher in non-polluted sites that is why farmed fish exhibited higher activity.

Catalase activity: Liver of *L. rohita* from a farmed habitat showed the highest while the least enzyme activity was found in gills of hatchery fish (Fig. 3).

Peroxidase activity: Peroxidase activity in different organs (liver, kidney, gills) of *L. rohita* is given in Fig. 4. Highest activity of peroxidase was observed in kidney (0.701 U mL^{-1}) of hatchery *L. rohita* and minimum activity was noted in the liver of farmed fish. The overall highest activity of peroxidase was observed in kidneys of *L. rohita* of all the three habitats. The activity of peroxidase in organs of *L. rohita* from different localities showed an increase in activity to formed the trend hatchery>river>farmed fish.

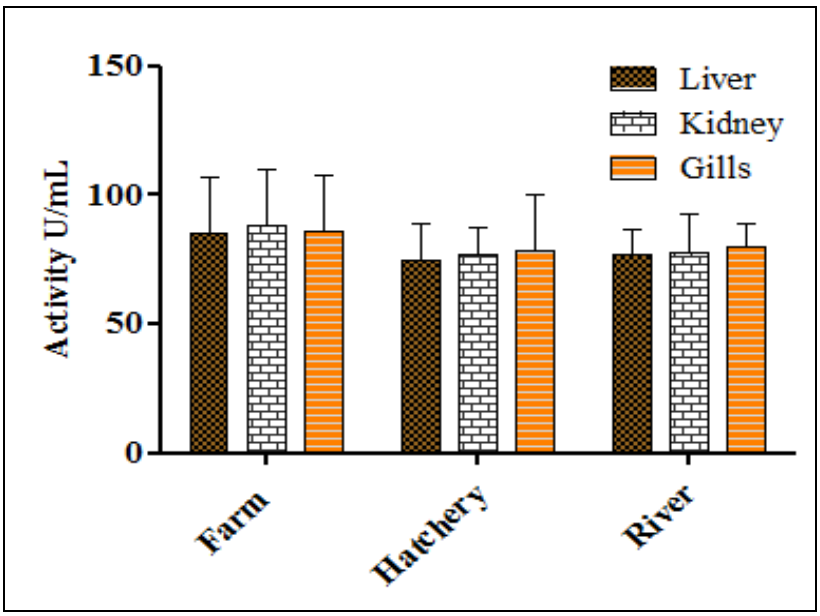

Figure 2: Superoxide dismutase activity in different organs of *Labeo rohita.*

Statistical analyses reveal that different enzyme (Superoxide dismutase, catalase, peroxidase) activity in *L. rohita* is highly significant at $p<0.05$ among sampling sites and fish organs, as well as the interaction between sampling sites and fish organs.

α-Amylase activity: Higher α- amylase activity was found in liver i.e. 7.75 U mL^{-1} of hatchery fish and lower in kidney (1.72 U mL^{-1}) of farmed fish (Fig. 5).

Mutarotase activity: Fig. 6 showed the mutarotase activity in liver, kidney and gills of *L. rohita*. Maximum and minimum mutarotase activity was found in liver (7.49 U mL^{-1}) of hatchery *L. rohita* and in gills (6.04 U mL^{-1}) of farm fish. However, the interaction between sampling sites and fish organs remained statistically at par.

Analysis of variance on mutarotase and α- amylase activity in fish showed significant differences at $p<0.01$ among sampling sites and no significant differences at $p>0.05$ among fish organs.

Determination of heavy metals

Zinc accumulation: The leading accumulation of zinc was observed in the kidney of river fish i.e. 13.11 µg/g and smallest amount in liver (3.12 µg/g) of farmed fish (Fig. 7). The maximum meditation of Zn was found in kidney and gills of *L. rohita.*

Copper accumulation: The maximum accumulation of copper was recorded in the kidneys (13.17 µg/g) of hatchery and minimum of 0.313 µg/g in the liver of farmed fish (Fig. 8). In the present research work, the maximum concentration of Cu was found in kidneys while liver and gills of *L. rohita* also exhibited a significant level. In kidney, the highest accumulation of metals altered the levels of biochemical parameters for heavy metal detoxification in the body.

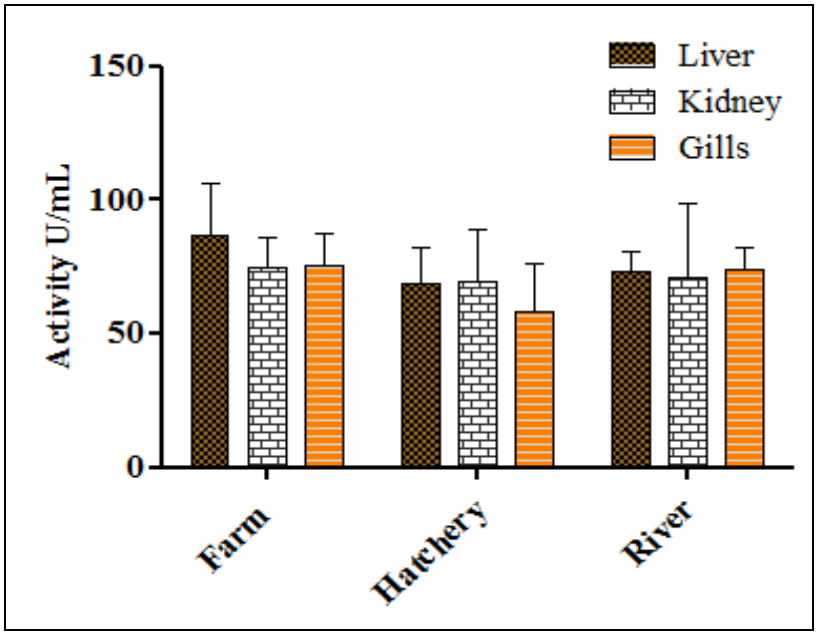

Figure 3: Catalase activity in different organs of *Labeo rohita.*

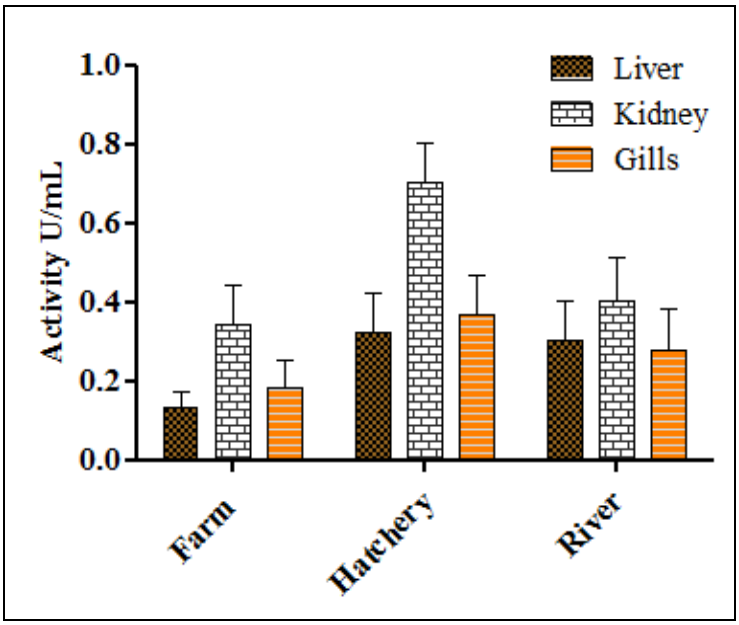

Figure 4: Peroxidase activity in different organs of *Labeo rohita.*

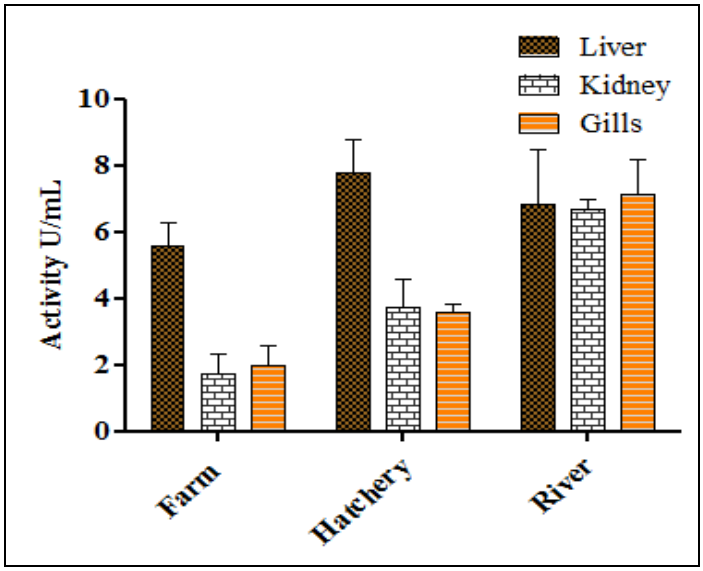

Figure 5: α- Amylase activity in different organs of *Labeo rohita.*

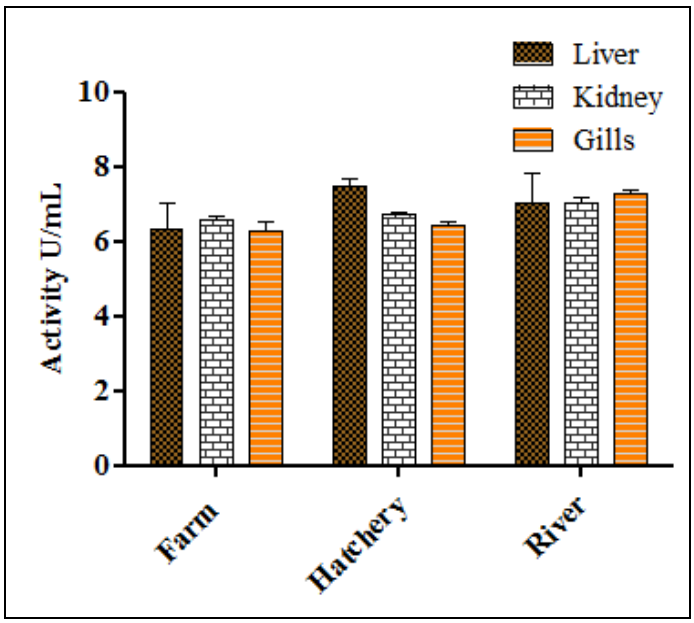

Figure 6: Mutarotase activity in different organs of *Labeo rohita.*

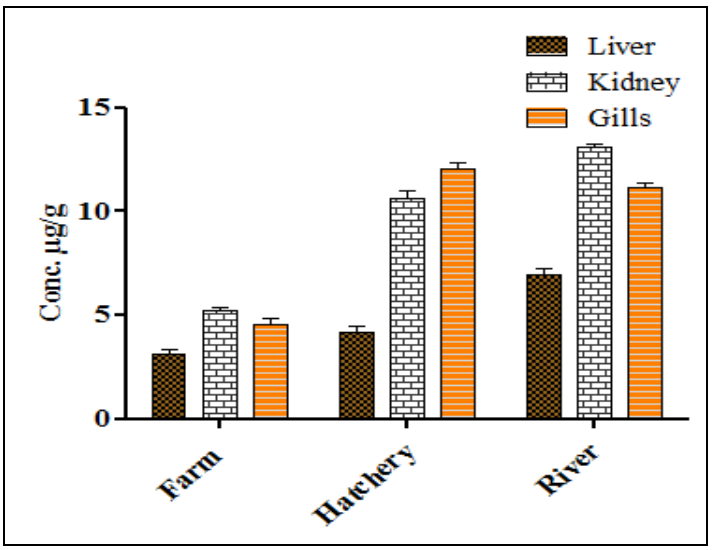

Figure 7: Concentration of Zn in different organs of *Labeo rohita.*

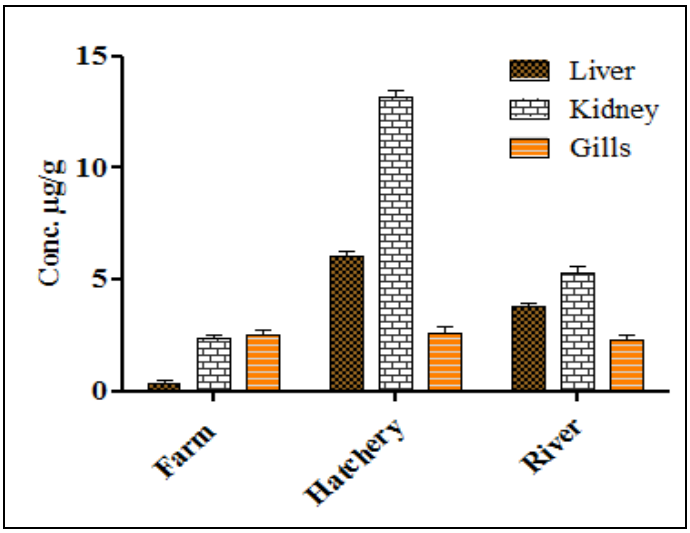

Figure 8: Concentration of Cu in different organs of *Labeo rohita.*

Nickel accumulation: The highest accumulation of nickel was observed in kidney of hatchery fish and the detailed results are shown in Fig. 9. The maximum concentration of Ni was found in liver and kidney of *L. rohita* whereas gills also showed a significant level.

Daily consumption safety

Our study demonstrated that the concentrations of heavy metal were in normal range expected for Ni. The dose of a toxic metal that one obtains from fish however, not only depends on the concentration of specific metals in fish, but also on the quantity of fish consumed. Although levels of heavy metals are not high, care must be taken considering some people regularly consume large quantities of fish.

Table 1: Standard levels and maximum range of concentrations of heavy metals (µg/g wet weight) in fish described in literature.

Organization/Country	Metals		
	Ni	Cu	Zn
FAO (1983)	-	30	30
FAO/WHO limits (1989)	-	30	40
Turkish guidelines (Dural *et al.*, 2007)	-	20	50
Range of metal in present study	2.25-3.43	0.313-13.17	3.12-13.11

All tissue concentrations are in µg/g wet weight.

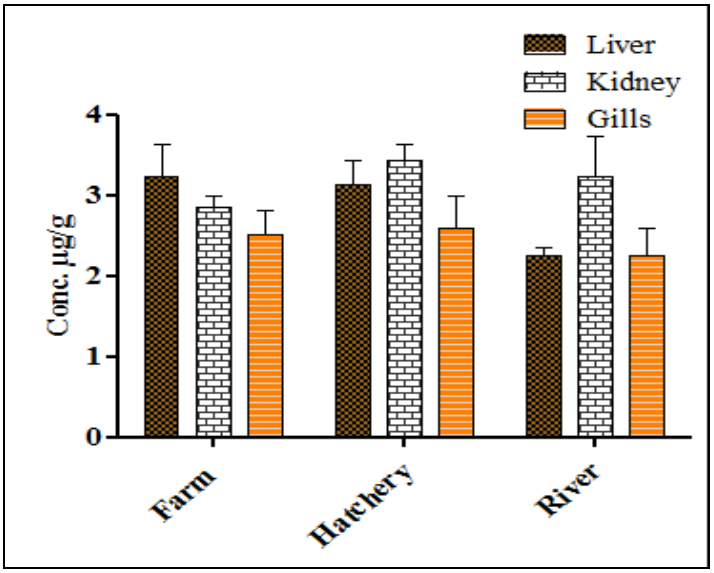

Figure 9: Concentration of Ni in different organs of *Labeo rohita* .

Genarally, in the present study, it was observed that in farmed fish the accumulation of heavy metals was not to a great extent and was considered suitable for consumers as compared to wild and hatchery fish. The meditation of heavy metals in kidney was highest than its corresponding values in other tissues. The metal accumulation was varied in different tissues within the same fish affecting the activity of antioxidant enzymes. Although levels of heavy metals are not high, care must be taken considering some people regularly consume large quantities of fish.

Discussion

Proximate analysis of fish meat samples

The maximum protein contents of fish were due to the good food consumption and its conversion into the protein in the fish meat as previously reported (Mahboob *et al*., 2004). It was observed that there was a significant increase in protein contents with an increase in body weight of fish.

Carbohydrates are also important but they are present in very less amounts in fish. The percentage of moisture is a good indicator to find lipids and protein contents in fish. The lower the percentage of moisture, the greater the lipids and protein contents (Dempson *et*

al., 2004). Vladau *et al*. (2008) reported that fish has protein contents in the range of 13 and 25%, which accounts for 80 to 90% of the energy content of the fish. The surveillance indicated that variations in proximate compositions of *L. rohita* were due to different habitats.

Analysis of antioxidant enzymes

Superoxide dismutase activity: Gills showed higher activity than liver and kidney tissues. Gills have direct contact with the different environmental factors and they are the primary route for the entry of toxicants, heavy metals and pesticides. According to Jordanoska *et al*. (2008) the superoxide dismutase activity differs from one species to another, from one tissue to another and from freshwater to marine environment as well. Depending on the degree of water pollution, fish have the ability to retain large amounts of pollutants in their tissues, either directly from the water by breathing or through their diet.

Catalase activity: Catalase activities may be decisively influenced by a number of environmental factors such as temperature, salinity, season and feeding habitats, age, sex. Shen *et al*. (2007) observed the catalase activity in Brocarded carp liver under pollutant exposure and the results showed that there is a decrease in catalase activity due to the fluctuation of superoxide radicals. Their findings agreed with the present research work, whereas the highest catalase activity was found in liver of farmed fish and then due to the accumulation of heavy metals the highly significant decrease in hatchery and wild fish. Presence of heavy metals showed inhibitory effect on catalase activity. McFrarland *et al*. (1999) reported that catalase activity was found lower in the contaminated black river fish as compared to the least contaminated environment.

Peroxidase activity: In fish, generally the kidney and liver have antioxidant defense systems to defend them from oxidative stress of heavy metals (Basha and Rani, 2003). Peroxidase activity was varying among the cells, tissues and organs, depending upon ecological conditions, feeding habitat and other environmental factors (Winston and Di Giulio, 1991).

The liver is a major site of detoxification and the first target of ingested oxidants and a very important tissue in the study of the role of peroxidase in protection from lipid peroxidation. Velma and Tchnounwou (2010) made an attempt to evaluate organ specific toxic effects of hexavalent chromium in kidney and liver of freshwater gold fish during acute exposure. Their results indicated that activity of peroxidase in both kidney and liver showed a significant increase and altered as compared to the control. Bray and Bettger (1990) observed the activity of peroxidase in liver of *G. brasiliensis* and results showed that the activity of enzymes is affected by the presence of zinc and copper potent activators.

α-Amylase activity: Change in digestive enzyme activity is affected by biochemical composition of feed and feeding behavior of fish. The digestive

process in fish is not well recognized as in mammals, although the data obtained in fish so far have shown that the digestive enzymes studied were qualitatively similar to those observed in other vertebrates.

Agrawal *et al*. (1975) determined the activities of digestive enzymes (amylase, sucrase, protease and lipase) in carnivorous fish *Wallago attu*, omnivorous fish *Clarias batrachus* and herbivorous fish *L. rohita*. The activity of amylase is higher in the *L. rohita* than in the *W. attu* and *C. batrachus*. The adaptations of the digestive system of different fish species exhibit correlation with their diet. This might be due to differences in their food components in both habitats.

Mutarotase activity: Bailey *et al*. (1969) studied the mutarotase content in kidneys of freshwater fish and saltwater fish. The results showed that the mutarotase content of kidneys in seven species of freshwater fish was significantly higher than in nine species of saltwater fish. There is no more literature available on the study of mutarotase in fish. The present research showed that mutarotase activity decreased in all organs of farmed fish as compared to that in hatchery and river fish.

Determination of heavy metals

Zinc accumulation: Zinc is an essential for body growth, maturation and development, tissue repair and resistance to disease.

It was concluded that exposure of heavy metals reduces the carbohydrate and lipid contents in muscles and gills in the three fish species. McFrarland *et al*. (1999) found that Zn is strictly connected to catalytic, structural and regulatory functions. It is a cofactor of superoxide dismutase and at the physiological level it has been required as a factor in protein synthesis for growth and development. Madhusudan *et al*. (2003) determined the bioaccumulation of zinc and cadmium in freshwater fishes.

Garg *et al*. (2009) studied the effects of exposure of zinc for 45 days on rohu fish. Zinc and copper accumulates mainly in metabolic organs liver and kidney which store metals for detoxification by producing metallothionein. Zn in certain concentrations is desirable for the growth of freshwater animals but its over accumulation is dangerous to exposed organisms as well as to those who consume them directly or indirectly through the food chain (Sultana and Rao, 1998).

Copper accumulation: Cu and Zn are essential for fish metabolism and gills act as the first line of defense and a prime site for metal accumulation whereas liver has the role in metal metabolism and detoxification (Jayakumar and Paul, 2006). Radhakrishnaiah (1988) reported that copper concentration increased significantly in the gills, liver, brain and muscle of *rohu*. Deb and Santra (1997) investigated the bioaccumulation of copper in the liver, intestine, ovary, muscles and brain in various fish. Copper accumulate in all fishes when

they are reliant on a sewage-fed ecosystem, in contrast to their freshwater control fish population.

Nickel accumulation: Nickel affects succinate dehydrogenase activity in liver, kidney, gills and muscles of *L. rohita* thereby inhibiting the energy yielding metabolic pathways. Moorthikumar and Muthulingam, (2011) observed the decreasing trend of succinate dehydrogenase activity in gills, liver, kidney, brain and muscle of fish, due to the effect of nickel.

Begum *et al*. (2009) reported the momentous level of nickel concentration in liver, kidney, gills and muscles of freshwater *L. rohita* and recorded the maximum concentration of heavy metals in kidney and liver. Abdullah *et al*. (2011) studied the metal bioaccumulation patterns in major carps during acute toxicity. Before Ni revelation, the *L. rohita* showed 3.58 µg/g concentration of nickel. Tariq *et al*. (1994) worked on commercially important fish species, along with relevant water and sediment samples, from non-polluted and polluted sites of the river Ravi and analyzed Cd, Cr, Cu, Ni, Pb, Zn and Hg by using atomic absorption spectrophotometer. The dispensation of trace metals in water, fish and sediment showed that the domestic and industrial effluents were responsible for the accumulation of metals in water at specific sites along the river.

Daily consumption safety

A large number of literature is available on heavy metal concentrations in fish, the majority of which were concerned either with different fish species collected from the same water body or with the same fish species collected from different localities. It is well known that, copper, nickel and zinc are essential elements, required by a wide variety of enzymes and other cell components and having vital functions in all living organisms, but very high intakes can cause adverse health problems (Demirezen and Uruc, 2006). It is also recommended to conduct continuous monitoring of commercial fish in Pakistan markets to ensure that the concentrations of metals remain within the prescribed worldwide limits.

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