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Assessing metal, protein, and DNA profiles in *Labeo rohita* from the Indus River in Mianwali, Pakistan

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Abstract This study assessed metals in water and different tissues of *Labeo rohita* and the impact of these metals on DNA and proteins as biomarkers of gills and muscles of these fish from three different polluted sites (reference or low = KW, medium = CH and high = SK) of the Indus River, Pakistan. The Mn, Pb, Cu, Zn, Hg, and Cr levels in water, gills, liver, muscles, and skin of these fish were compared with the international permissible levels. All metals except Pb and Hg in water were within the acceptable limits of drinking water. In contrast, the Mn, Hg, and Cr levels in the fish tissues were higher than their permissible limits for fish as a human food. Here, the gills contained higher metals than the other tissues. Different patterns of biomarkers were found in fish from these sites. While the gills did not show four protein bands (55, 30, 18.4, and 16.4 kDa), the muscles showed four new protein bands (100, 85, 45, and 20 kDa) for fish from the medium and high polluted sites as compared to the reference or low polluted site. The fish from the CH and SK sites of the Indus River contained low molecular weight DNA in their gills but high molecular weight DNA in their muscles when compared with the KW site. This study suggests that the proteins and DNA profiles of *L. rohita* could be used as biomarkers to assess the impact of potential environmental stressors such as metals on the freshwater systems.

Keywords *Labeo rohita* · Metals · Indus River · SDS-PAGE · Proteins · DNA · Biomarkers

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Introduction

Urban streams are one of the ecosystems most affected by the contamination due to human activity (Paul and Meyer 2001). Agricultural, industrial and domestic effluents containing various organic and inorganic pollutants, such as solvents, oils, heavy metals, pesticides, and fertilizers, etc. are, invariably, discharged into small rivers and streams without their proper treatments (Pandey et al. 2003). Such contaminants change water quality and may cause diseases and structural alterations in fish (Chang et al. 1998).

The widespread metal contamination is worrying because of its toxicity, persistence, and bioaccumulative nature. In the fluvial environments,

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metals are produced from atmospheric deposition, geologic weathering, and numerous agro-industrial and residential activities (Demirak et al. 2006). To assess the overall quality of the aquatic environment, different biological responses can be examined to estimate the impact of pollutants. In fact, the changes in biological functions, structures and proteins in response to metal pollution may be used to assess the fish health as an early warning signal of various environmental risks (Van der Oost et al. 2003; Cajaraville et al. 2000; Newman 1998). However, there is no single biomarker that can unequivocally measure the environmental degradation. This problem is solved by the use of a set of complementary biomarkers, a methodological approach that is now widely recognized for environmental biomonitoring (Galloway et al. 2004).

Our previous studies from the same area of Indus River showed high metal levels in different fish tissues especially in the edible parts of *Oreochromis mossambicus* and *Cyprinus carpio* (Jabeen and Chaudhry 2009a, b). This aspect may impact on the health and livelihood of the neighboring communities which also involve fishing for *Labeo rohita* as another valuable and fast growing fish of this area. Therefore, this study assessed the metal concentration in water and different fish tissues and their effects on protein and DNA profiles of gills and muscles of *L. rohita,* as another bioindicator. This information could be integrated with different early warning systems to monitor freshwater pollutions and subsequently to adopt practices to avoid their impacts on the aquatic and human populations.

Materials and methods

Study area and its significance

The study was conducted in the Mianwali District of Pakistan, which is located along the bank of the Indus River. Mianwali is one of the northwestern cities in the province of Punjab, Pakistan with an area of 5,840 km^2. The city is located at 32.58° (latitude) and 71.55° (longitude) with an altitude of 209 m. This district is rich in minerals, clay, coal, gypsum, limestone, salts, and rocks which are commercially excavated as previously reported by Jabeen and Chaudhry (2009a, b). The inland fisheries in Pakistan are heavily dependent upon the Indus River, which is one of the world's largest rivers for its drainage basin area (970,000 km^2), discharge and sediment load as it flows from the Northern mountains to the Southern plains before it falls alongside other rivers into the Indian Ocean in the Sindh province. This study covered three sites around the Indus River in the Mianwali District. Here, Kukranwala (KW) site was designated as reference or low polluted site due to relatively less human activities in its surrounding. The other two sites called Shehbaz Khel (SK) and Chashma (CH) were designated as heavy and medium polluted sites, respectively, as these sites were receiving different amounts of pollutants from their adjacent areas. Therefore, this study investigated the effect of pollution on metal concentrations in water alongside different fish tissues. The impact of these metals on the protein profiles and DNA integrity of gills and muscles of *L. rohita* from these sites were also studied. These metals (manganese = Mn; lead = Pb; copper = Cu; zinc = Zn; mercury = Hg, and chromium = Cr) were selected because Mn, Cu, and Zn are essential for fish metabolism whereas, Pb, Hg, and Cr are regarded as highly toxic and yet their role in fish biology is mostly unknown. Here, fish muscles and skin were selected as edible components, the gills as the first line of defense and a prime site for metal accumulation (Jayakumar and Paul 2006) and liver for its role in the metal metabolism and detoxification.

Water sampling and preservation

Representative samples of about 1 l water were collected in polypropylene bottles that were washed with distilled water and then with the river water from the sampling sites. The water samples were collected in September, 2008 at midday from three locations as three replicates from each site at around 30 cm depth. These water samples were filtered and preserved in 5 ml of 55% HNO_3/l of water to prevent metal adsorption on the inner surface of the container and stored at 4°C before their analyses as described below.

Metal analysis of water samples

Each 100 ml of preserved water sample was evaporated in a volumetric flask on a hot plate to about 20 ml within a fume cupboard. After cooling, 5 ml HNO_3 (55%) and 10 ml perchloric acid (70%) were added. The mixture was evaporated on a hot plate until the brown fumes were converted into dense white fumes of perchloric acid. The sample was cooled and diluted to 100 ml with distilled water. The solutions were then aspirated into an atomic absorption spectrophotometer (model AA-660X VI42, Varian Ltd.) by using an air–acetylene flame for the determination of these metals. Standard solutions of relevant metals were used to construct standard curves for their comparisons with the sample readings to determine each metal concentration. The fish samples were analyzed at the Newcastle University by using ICP-OES as described later.

Fish sampling and morphometric observations

The fish samples were collected in September 2008 concurrently with the water sampling. The fishing was performed by using traditional gill nets in coordination with the local fishermen as previously described by Jabeen and Chaudhry (2009a, b). The total fish catches were harvested from three nets per site and the relevant live fish samples of similar size were transferred to large water buckets and the remaining fish were sold. These fish were then quickly killed by using the concussive blow to their heads (percussive stunning). Twenty seven samples of *L. rohita* by involving nine fish per net as replicates were collected on ice from each site. The fish samples were immediately transported to the laboratory where morphometric measurements by involving fresh dead weight (FDW), length, and width of each fish were carried out.

Fish dissection and preservation

After morphometric measurements, each fish was dissected to collect gills, skin, liver, and muscles. These organs were weighed individually, washed with distilled water, drained on filter papers, transferred into sterilized polythene bags and stored in a freezer at −20°C until further analysis.

Metal analysis of fish tissues

The frozen fish tissues were carried to the UK in July 2009 and stored at −20°C until freeze-dried and ground afterwards. For metal analysis, 1 g of each dried sample was digested in 10 ml of concentrated HNO_3 (VWR, UK) by using digestion blocks at 80°C. Each digested sample was evaporated to about 2 ml, cooled, diluted to 10 ml with distilled water and filtered with Whatman filter paper 1. These samples were then analyzed by using inductively coupled plasma optical emission spectroscopy (ICP-OES, Unicam 701). The machine was calibrated over the relevant concentrations using individually certified standards from Sigma Aldrich, UK. The metal concentrations in different fish tissues were reported as mg/kg dry matter (DM), because DM rather than wet weights (WW) provides a more stable basis for comparison. However, for comparing these metal concentrations with international standards which are presented on WW basis, we converted the metal concentration data from DM to WW basis by using the following formula:

$$\text{WW concentration} = (\%\ \text{DM concentration}) \times (1 - \%\ \text{moisture content}/100)$$

Protein and DNA profiles for biomarker analysis

Fifteen fish samples from each site were selected to determine the protein profiles and DNA integrity in their gills and muscles. The gills were analyzed for biomarker responses because the highest metal load was observed in gills than other tissues of this study and muscles were analyzed because of their prime use as a quality food for human consumption.

Chemicals and preparation of fish samples for protein extraction

All chemicals were purchased from either Fisher UK Ltd, or VWR or Sigma Aldrich UK Ltd. unless otherwise stated in the following sections. About 0.1 g samples of freeze-dried fat-free fish

muscles and gills were separately mixed in 1.5 ml of 1× sodium dodecyl sulfate (SDS) sample buffer at pH 6.8 (0.0625 M Tris–HCl, 2% SDS, 10% *v*/*v* glycerol, 0.1 M mercaptoethanol, 0.01% bromophenol blue) and 7.5 µl of protease inhibitor cocktail. The mixture was heated to 95°C for 3 h in a shaking water bath and then centrifuged at 14,000×*g* for 30 min. Each supernatant containing protein extract was carefully transferred to a dialysing membrane (Spectra/Pro, MWCO 3.5 kDa, Spectrum) for dialysis in poly ethyl glycol (PEG 1000). The protein concentration of each sample was then determined by the Bradford (1976) method. The protein samples were stored at −20°C until their use for electrophoresis.

SDS-polyacrylamide gel electrophoresis

The SDS-polyacrylamide gel electrophoresis (PAGE) gradient gel system was used as suggested by Laemmli (1970) for the evaluation of protein fractions in the gill and muscle tissues of these fish by using the dual TV400 twin-plate maxi-gel vertical system (VWR, UK). For this purpose 12–20% resolving gel [30% acrylamide, 0.8% *N*,*N* methylene bisacrylamide in 1.5 M Tris–Cl, pH 8.8, 10% SDS, 10% Ammonium persulfate (APS), 6.6 µl *N*,*N*,*N*,*N*-tetramethylenediamine (TEMED)] with a 3% stacking gel (10% acrylamide:2.5% bisacrylamide in 0.5 M Tris–Cl, pH 6.8, 0.46% TEMED, 10% SDS, 10% APS) was used. The electrode buffer contained Tris, SDS, and glycine (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3). Standard peQ Gold protein markers I (MW 14.4–116 kDa) and II (MW 10–200 kDa) from peQLab, UK were used. Before their loading, protein samples and standards were heated in a water bath at 95°C for 5 minutes and then centrifuged at 10,000×*g* for 5 min. Supernatants were collected and only 5 µl of each sample and protein standards were loaded into each well and the gel was run first at 50 V for 1 h and then at a constant current of 120 V for around 10 h to obtain visible bands on the gel.

Gel staining with Coomassie brilliant blue R

Each gel was stained by adapting the solution for fast visualization of protein—US Patent 6555382. After electrophoresis, the gel was immersed in about 200 ml of deionized water in a tray which was boiled in a microwave oven. The liquid was drained and the gel was washed for about 5 min with deionized water which was also removed afterwards. About 100 ml of a staining solution containing 0.006% Coomassie brilliant blue R in 35 mM HCl solution was added to the gel which was boiled in a microwave oven and the gel was kept in the staining solution for another 5 min. The staining solution was then removed, and the gel was rinsed with deionized water to view the stained proteins as intense blue bands which were then photographed by a digital camera.

DNA extraction from fish tissues

The extraction of DNA from the fish gills and muscles was performed with a phenol/chloroform method by using autoclaved glassware and equipment. About 15 mg samples of ground freeze-dried tissues were mixed in a DNA extraction buffer (250 mM NaCl, 100 mM EDTA and 100 mM Tris base) and the cells were lysed by adding 10% sodium lauryl sarcosinate. The RNA was removed by adding RNAse (10 mg/L) followed by their incubation on ice for 10 min. Proteinase K was added (1 mg/L) to remove protein and the samples incubated overnight at 55°C in a shaking water bath. The extracts were further purified by extracting twice with phenol:chloroform:isoamyl alcohol (25:24:1 *v*/*v*) and finally with chloroform. Each time, equal volumes (500 µl) of these solvents were mixed with relevant samples before their centrifugations at 10,000×*g* for 10 min at room temperature. The upper aqueous layer was transferred into a new micro-centrifuge tube to which 30 µl (1/10th volume) of 3 M sodium acetate at pH 5.2 were added to each sample. Two volumes of 100% chilled ethanol were then added to each sample and mixed well until DNA was visible after which these samples were kept for DNA precipitation at −20°C for 12 h. These samples were then centrifuged at 15,000×*g* for 10 min at 4°C. The pellet was washed with 70% ethanol; air dried, and finally re-suspended in 60 µl sterilized deionized water. Optical density (OD) of each sample was measured at 260 and 280 nm by

Table 1 Mean metal concentration in water at different sites of the Indus River and permissible levels of these metals according to WHO, USEPA, and NEQ Standards

Metals (mg/l)	This study sites			WHO[a]	USEPA[b]	NEQ[c]
	Reference site (KW)	CH	SK			
Mn	0.013 ± 0.001^{a}	0.023 ± 0.001^{b}	0.024 ± 0.001^{b}	0.5	0.05	1.5
Pb	0.073 ± 0.02^{a}	0.183 ± 0.02^{b}	0.237 ± 0.01^{c}	0.01	0	0.5
Cu	0.147 ± 0.01^{a}	0.282 ± 0.01^{b}	0.342 ± 0.02^{c}	2	1.3	1
Zn	0.273 ± 0.02^{a}	0.350 ± 0.01^{b}	0.400 ± 0.03^{c}	3	5	5
Hg	0.008 ± 0.001^{a}	0.023 ± 0.007^{b}	0.025 ± 0.01^{c}	0.001	0.002	0.01
Cr	0.037 ± 0.001^{a}	0.151 ± 0.01^{b}	0.170 ± 0.01^{c}	0.05	0.1	1

Means with the same capital letters in the same row did not differ significantly, $P > 0.05$
[a]WHO guidelines: http://www.who.int/water_sanitation_health/dwq/en/dgwq3_ann4tab.pdf
[b]US standards: http://www.epa.gov/safewater/contaminants/index.html#inorganic
[c]NEQ standards: National Environmental quality standards, Pakistan (1999)

UV-spectrophotometer (Biochrom Libra S12) and the purity of DNA was measured by the OD_{260}/OD_{280} ratio (ideal ratio = 1.7–2.0).

Agarose gel electrophoresis and staining for DNA

The purified DNA extracts together with a marker (DNA sizer II) containing eight fragments (23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, and 125 bp) from peQLab UK, were loaded on a non-denaturing 0.5% agarose gel in TBE buffer (45 mM boric acid, 2.5 mM EDTA, 135 mM Tris base, pH 8). Bromophenol blue was added to each sample before its loading in each gel to mark the migration of these extracts. Gel electrophoresis was carried out at 70 V for around 1 h using a horizontal mini gel electrophoresis system (VWR, UK). The migrating DNA was observed by staining the gel for about 40 min in the dark in 200 ml TBE buffer and 20 µl SYBR Green I. The stained gels were captured by using Gene Snap Version 4.01.00 of Syngene. The migration distance of the DNA molecules from the top of the gel was used as a measure of DNA damage. The migration of the DNA is inversely proportional to the length of the DNA molecules and so it was used to estimate the distribution of different strand lengths (fragments) together with the relative mobility (Rf) value. Where Rf = the distance from the origin to the centre of each fragmented DNA band on the gel divided by the total distance travelled by the tracking dye from the top to the bottom of that gel. It is recognized that the highly fragmented, low molecular weight DNA strands will migrate farther than non damaged high molecular weight DNA strands. The gel photographs were used to estimate the Rf values of DNA fragments of these fish gills and muscles. The DNA fragments and their Rf values were then compared with those of the DNA markers to determine the effect of river water environment on the DNA of these fish.

Table 2 Mean length, width, fresh dead weight (FDW), and weights, as % of FDW, of muscles, skin, gills, and liver in *L. rohita* from the Indus River

Parameters	Study sites			SE	P-Value
	Reference site (KW)	CH	SK		
Length (cm)	45.72 ± 0.46	43.86 ± 0.64	44.02 ± 0.68	0.74	0.653
Width (cm)	13.53 ± 0.09	12.27 ± 0.12	12.44 ± 0.26	0.22	0.172
FDW (g)	833.3 ± 16.7	783.3 ± 16.7	791.7 ± 22.1	22.89	0.208
Organs as % FDW muscles	65.33 ± 0.94	64.74 ± 1.3	60.87 ± 1.5	1.54	0.089
Skin	6.66 ± 0.22	6.50 ± 0.26	6.86 ± 0.45	0.40	0.744
Gills	4.70 ± 0.12	3.83 ± 0.47	4.59 ± 0.18	0.36	0.161
Liver	0.66 ± 0.02	0.59 ± 0.10	0.58 ± 0.09	0.10	0.751

Statistical analysis

The data were statistically analyzed by general linear model by using Minitab software to compare the main effect of either only the sampling sites on water and fish parameters or the sampling sites, fish organs and their site × fish organ interaction on the mineral profiles of these fish organs. These effects were declared significant if $P < 0.05$ and highly significant if $P < 0.01$. Tukey's post-hoc test was used to compare means. However, no statistical analyses were performed for the protein and DNA profiles of fish gills and muscles of this study. Microsoft Excel 2007 was used to present means of individual metals as graphs for comparing different fish tissues of this study.

Results

Metal profiles of water

Table 1 presents the mean values of metals in water at selected sites of the Indus River and the permissible guide lines for these metals by the international and national standards. All metals except Pb and Hg were within the permissible limits at all the sites of the Indus River. However, the metal concentrations at low polluted site (KW) were lower than the medium and high polluted sites (CH and SK). Generally, metal concentrations at SK were higher than CH.

Morphometric parameters and metal concentrations of fish

Table 2 shows the mean values of length, width, fresh dead weight (FDW), and weight as percentage of FDW of muscles, skin, gills, and liver in *L. rohita* from three sites of the Indus River. There was no significant difference among three sites for any of the selected fish parameters ($P > 0.05$).

Table 3 shows the mean concentration of metals in different organs of *L. rohita* for each of the three sampling sites of the Indus River alongside the relevant SE and significance for the main effects of the sites and organs and their interaction. All metals showed highly significant

Table 3 Mean metal concentration (mg/kg DM + SD) in different organs of *L. rohita* from different sites of the Indus River together with SE and significance

Metals	Gills			Liver			Muscles			Skin			SE and significance		
	KW	CH	SK	KW	CH	SK	KW	CH	SK	KW	CH	SK	Site (S)	Organ (O)	S x O
Mn	22.52 ± 0.02	57.95 ± 0.40	62.28 ± 0.09	2.30 ± 0.10	5.08 ± 0.03	5.10 ± 0.04	0.18 ± 0.01	3.05 ± 0.72	4.18 ± 1.08	0.64 ± 0.01	2.12 ± 0.01	1.70 ± 0.01	0.20***	0.23***	0.20***
Pb	1.15 ± 0.01	4.94 ± 0.08	5.20 ± 0.08	0.00 ± 0.00	1.05 ± 0.46	1.23 ± 0.14	0.00 ± 0.00	2.26 ± 0.07	1.48 ± 0.51	0.21 ± 0.04	2.01 ± 0.05	1.11 ± 0.03	0.10***	0.12***	0.10***
Cu	2.39 ± 0.01	7.29 ± 0.01	5.24 ± 0.02	21.11 ± 0.22	441.7 ± 3.5	577.87 ± 3.38	1.93 ± 0.02	3.75 ± 1.21	2.23 ± 0.35	1.95 ± 0.03	4.16 ± 0.01	1.97 ± 0.01	0.72***	0.84***	0.72***
Zn	137.9 ± 0.55	423.9 ± 1.8	263.7 ± 0.3	131.7 ± 0.36	101.6 ± 1.4	220.6 ± 3.23	17.8 ± 0.44	21.1 ± 2.00	21.5 ± 2.51	25.2 ± 0.15	99.4 ± 0.15	27.0 ± 0.13	0.75***	0.86***	0.75***
Hg	1.13 ± 0.05	2.90 ± 0.07	2.67 ± 0.03	1.39 ± 0.24	4.11 ± 0.45	3.79 ± 0.14	0.22 ± 0.10	2.54 ± 0.25	2.17 ± 0.11	0.28 ± 0.09	1.81 ± 0.03	1.4 ± 0.02	0.09***	0.10***	0.09***
Cr	1.59 ± 0.04	4.48 ± 0.06	3.32 ± 0.04	1.19 ± 0.05	3.18 ± 0.03	2.30 ± 0.07	0.13 ± 0.02	1.76 ± 0.27	1.12 ± 0.11	0.34 ± 0.01	1.56 ± 0.02	0.51 ± 0.03	0.05***	0.05***	0.05***

SD standard deviation, *SE* standard error
*** $P < 0.001$

differences in accumulation patterns among sites, organs and site × organ interactions ($P < 0.01$). Low metal loads were observed in the organs of fish sampled from low polluted area (KW) than those from the sites receiving domestic and municipal wastes and agriculture runoffs (CH and SK; Table 3). Metal concentrations in different tissues of fish from the KW site were in the order of gills > liver > skin > muscles whereas, in fish from the CH and SK sites were in the order of liver > gills > skin > muscles. In fact, Mn, Pb, Zn, and Cr showed highest accumulations in gills whereas Cu and Hg showed highest concentrations in liver at all these sites of the Indus River (Table 3). It appeared that the gills were more susceptible to metal contamination followed by liver, skin, and muscles. Thus the overall order of metal concentrations in selected tissues of *L. rohita* from the Indus River was gills > liver > skin > muscles. Figure 1 showed the mean concentrations of different metals in gills, liver, muscles, and skin and their level of significance in these organs of *L. rohita* from the Indus River.

The moisture contents of fish muscles were evaluated to enable the like with like comparison of metal concentrations reported on both DM

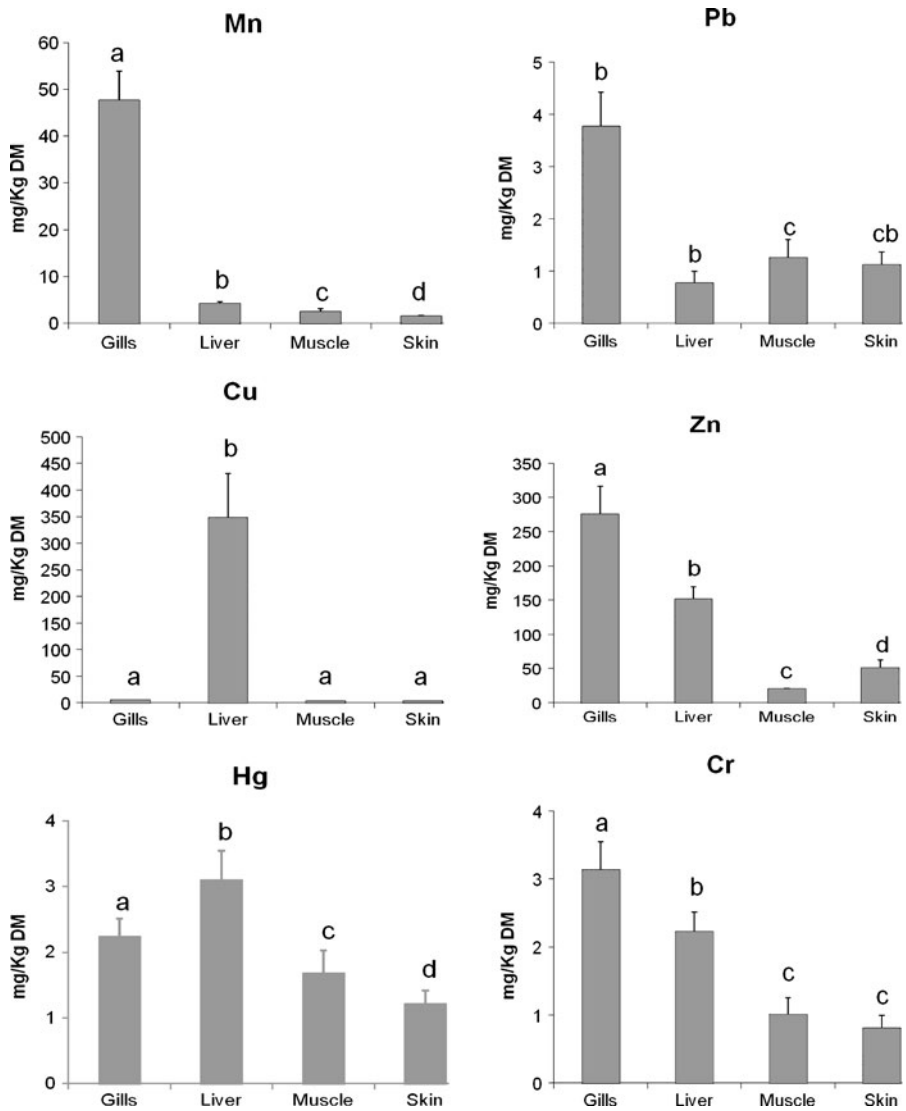

Fig. 1 Mean metal concentrations in mg/kg DM in gills, liver, muscles, and skin of *L. rohita* together with standard errors of each mean as *error bars*. Here, means as columns showing *different letters* differ significantly at $P < 0.05$

WW basis. Overall, the moisture contents in the muscles of *L. rohita* from the Indus River ranged between 78% for SK and 80% for both the KW and CH sites.

Protein profiles of the fish gills and muscles

Figures 2 and 3 represent the protein fractions in the gills and muscles of *L. rohita* from the KW, CH, and SK sites of the Indus River. Different patterns of protein fractions were observed in the fish tissues of the KW site (low polluted) than the CH and SK sites (medium to high polluted). While the gills of fish from CH and SK sites showed four major proteins, the fish from the KW site showed

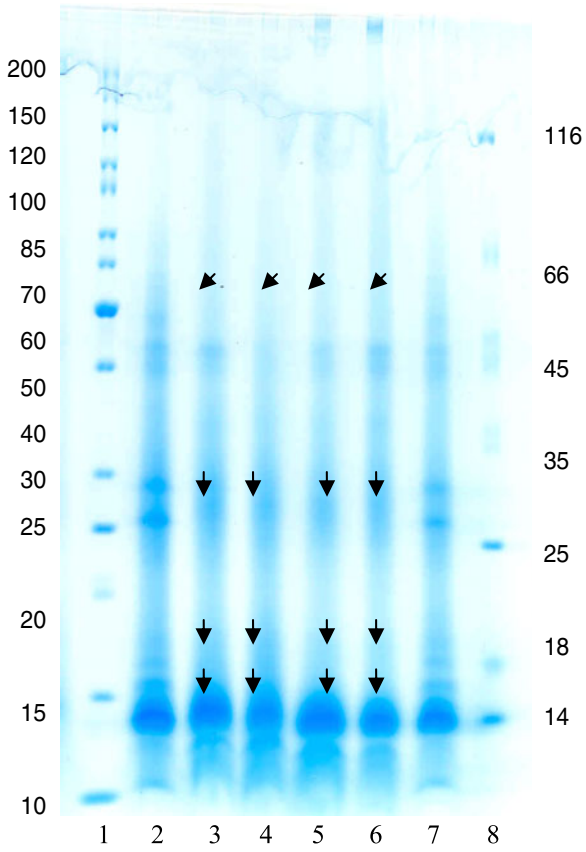

Fig. 2 Protein fractions (KDa) of gills of *L. rohita* from polluted (CH and SK) (*3–6*), and reference (KW) (*2*, *7*) sites of the Indus River alongside high (*1*) and low (*8*) molecular weight markers in KDa. *Arrows* indicate the disappearance of various bands from the gills of fish from the polluted sites

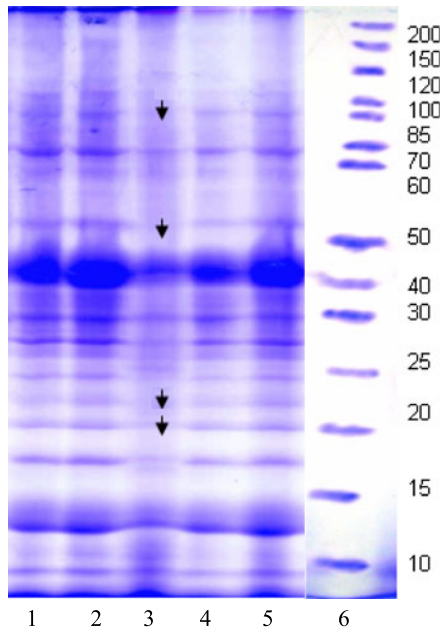

Fig. 3 Patterns of muscle proteins (KDa) of *L. rohita* from the polluted (*tracks 1*, *2*, *4*, *5*) and reference site (*track 3*) of the Indus River alongside the protein marker (*track 6*). *Arrows* indicate the bands which were absent from the reference site (KW) fish but appeared in the samples from the polluted sites (CH and SK) of the Indus River

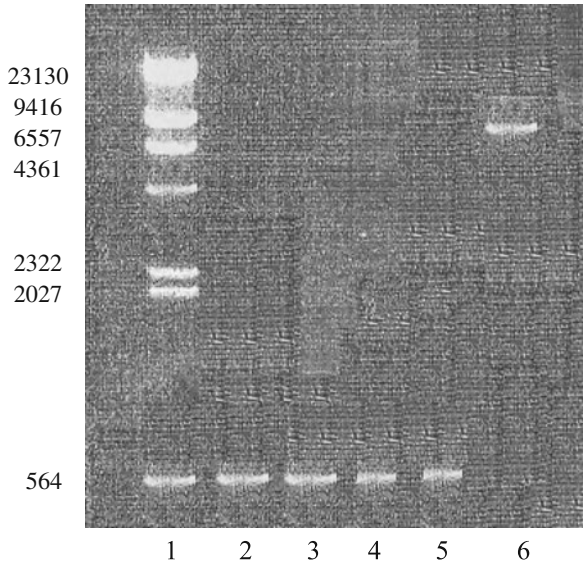

Fig. 4 DNA marker (*track 1*) and DNA extracted from the gills of *L. rohita* from the polluted (*tracks 2–5*) and reference (*track 6*) sites of the Indus River

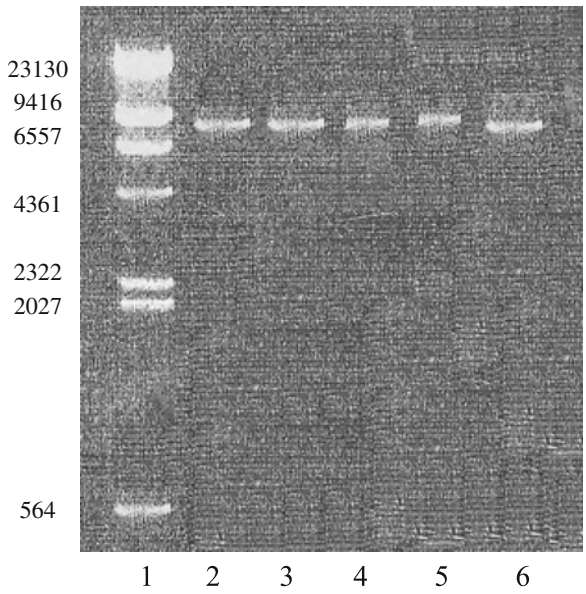

Fig. 5 DNA marker (*track 1*) and the DNA extracted from muscles of *L. rohita* from the polluted (*track 2–5*) and reference (*track 6*) sites of the Indus River

eight major proteins. The fish muscles from the CH and SK sites contained 14 major proteins whereas only ten were observed in fish from the KW site.

DNA profiles of fish gills and muscles

Figures 4 and 5 show the gel photographs of a DNA sizer II marker and the DNA from the fish muscles and gills from three sites of the Indus River. The muscle DNA of fish from the KW, CH, and SK sites did not differ in integrity. All fish muscle DNA showed similar patterns of migration and Rf values and hence showed same molecular weight of about 7,986 bp (Fig. 4; Table 4). However, the gill DNA for the CH and SK sites differed markedly in their migration from those for the KW site (Table 4; Fig. 5). The KW fish showed the DNA of high molecular weight of about 7,986 bp with Rf value of 0.262; whereas the gill DNA for the CH and SK sites of the Indus River showed lower molecular weight of about 564 bp with a higher Rf value of 0.876 (Table 4).

Discussion

Metal profiles of water

All metals except Pb and Hg in water from our study sites were within the safe limits as suggested by the International standards for water quality. Higher levels of Pb and Hg even at the low polluted site of the Indus River than those of the International standards were worrying because of their potential toxicity for the aquatic organisms. Low levels of metals at the low polluted site than other two sites may be attributed to less human activities involving no nearby cultivation and its long distance from the major sources of contamination for this river. High levels of Hg and Pb in the Indus River water of Mianwali District may also be attributed to its tributaries which dump unregulated industrial effluents and municipal wastes into this

Table 4 Migration distance (mm) and Rf values for the marker and fish sample DNA imaged on the gel together with their estimated fragment sizes in base pairs (bp)

Items	Migration	Rf value	Fragment size
Marker 1	6	0.15	23130
Marker 2	9	0.225	9416
Marker 3	12	0.3	6557
Marker 4	13.5	0.337	4361
Marker 5	20	0.5	2322
Marker 6	22	0.55	2027
Marker 7	35	0.875	564
Gill DNA from KW site	10.5	0.262	7986
Gill DNA from CH & SK sites	35	0.875	564
Muscle DNA from KW site	10.5	0.262	7986
Muscle DNA from CH & SK site	10.5	0.262	7986
Total distance of tracking dye from the start to end points	40		

river. This agreed well with the findings of Tariq et al. (1996), who reported high metal pollution in fish, sediments, and water from this part of the Indus River even over 10 years earlier than this study.

Morphometric parameters and metal concentration of fish

Almost similar characteristics of fish from the selected sites confirmed that the selected fish were almost uniform in their morphometric measurements. This study showed much higher levels of Mn (1.7–62.2 mg/kg DM) in fish organs from the CH and SK sites than those (0.18–22.5 mg/kg DM) of the KW site which was confirmed for its low pollution. Overall Mn concentration of 0.18–4.18 mg/kg DM = 0.037–0.86 mg/kg WW in fish muscles from all selected sites of the Indus River were much higher than 0.01 mg/kg WW of the WHO (1985) standards. More Mn concentration was recorded in the gills than the other organs of this study, indicating more affinity of Mn accumulation in gills than liver, muscles and skin. This agreed well with the findings of Jabeen and Chaudhry (2009a) who reported higher metal loads in gills than muscles of *O. mossambicus* from the same location of the Indus River. Mn is an essential micronutrient (Dallas and Day 1993) as it functions as a cofactor for many enzyme activities (Sures et al. 1999). Mn can be taken up directly through the gills or indirectly from food and ingested sediments via gut (Bendell-Young and Harvey 1986) which is supported by this study. High Mn concentration interferes with the central nervous system of vertebrates by inhibiting dopamine formation as well as interfering with other metabolic pathways such as Na regulation which ultimately can cause death. High Mn levels in gills and liver (non-edible) or muscles and skin (edible part) are a matter of concern as the consumption of Mn contaminated fish could result in the Mn-related disorders in the consumers.

Pb is considered as a toxic but non-essential metal implying that it has no known function in the biochemical processes (Adeyeye et al. 1996). However, it is acknowledged that lead enters the aquatic environment through soil erosion and leaching, gasoline combustion, municipal, and industrial wastes and runoff (DWAF 1996). The Pb concentrations (1.48–2.26 mg/kg DM = 0.30–0.47 mg/kg WW) in the fish muscles for the CH and SK sites of this study were within the maximum acceptable limit of 2 mg/kg for food fish (WHO 1985; FEPA 2003). Therefore, *L. rohita* from the Indus River should be safer for human consumption in relation to their Pb levels as Pb accumulated more in gills than other organs of these fish.

Cu being a constituent of many enzymes affects growth and reproduction of fish. So its maximum accumulation in the livers of fish of this study was perhaps due to the greater tendency of metals to react with the oxygen carboxylate, amino group, nitrogen, and/or sulfur of the mercapto group in the metallothionein protein which was highest in the liver (Al-Yousuf et al. 2000; Ikem et al. 2003). Overall Cu concentration in the muscles of *L. rohita* from the Indus River (1.93 to 3.75 mg/kg DM = 0.40 to 0.77 mg/kg WW) was within the permissible limits of 30 mg/kg WW (WHO 1985; FEPA 2003). Zn is an essential element in an animal's diet but it is regarded as a potential hazard for both animal and human health (Amundsen et al. 1997). Overall Zn concentration in muscles of *L. rohita* from the Indus River (17.8–21.5 mg/kg DM = 3.68–4.44 mg/kg WW) was much lower than the permissible levels of 50, 75, and 40 mg/kg WW (WHO 1985; FEPA 2003). Consequently, the fish from the Indus River is safer for human consumption in relation to the zinc levels despite its greater affinity of accumulation in the gills than other fish organs which agreed well with the findings of Sidoumou et al. (2005). Such higher concentrations of Cu and Zn in gills than muscles could be due to the high volumes of water being filtered through the gills.

Mercury is a highly toxic and is the most closely monitored contaminant in fish which are known as one of the single largest mercury source for human beings. In addition to its natural occurrence, the Hg levels have been greatly increased by the anthropogenic sources such as incinerators, coal-burning (electrical generation) and industrial production of chlorine, caustic soda, batteries, fluorescent lights, and thermometers, etc.

(UNEPGEMS 2006). The high levels (0.22 mg/kg DM = 0.044 mg/kg WW) in fish muscles from the KW site were much lower than 0.14 mg/kg WW of WHO standards (1985) and so the fish from this site could be safe for human consumption. Conversely, the Hg concentration (2.17–2.54 mg/kg DM = 0.44–0.52 mg/kg WW) in the fish muscles from the CH and SK sites exceeded the permissible levels of WHO (1985) for edible fish. In humans, prenatal exposure to high Hg levels, particularly in fish-eating populations, are associated with the central nervous system related problems (WHO 2004). These investigations match well with the previous findings where high Hg levels were found in different organs of *O. mossambicus* from the same area of the Indus River (Jabeen and Chaudhry 2009a). The higher Hg levels in liver than muscles of this study indicated that the fish from the Indus River were contaminated with Hg from the river water. Many earlier studies (Svobodová et al. 1995; Foster et al. 2000; Linde et al. 2004; Havelkova et al. 2008) also indicated that the fish from heavily contaminated localities deposited Hg preferentially in the liver, while in slightly contaminated areas Hg was deposited preferentially in muscles. These investigations are in line with the present findings where Hg levels in water of the Indus River at all selected sites exceeded the safer water quality criteria of the WHO and USEPA standards (Table 1). As mercury could be highly toxic for the behavior of vertebrates, enzyme activity, and cell division, it is vital to investigate Hg contamination in freshwater fish for the fish in order to protect the health of the fish and the relevant consumers.

Chromium is a relatively scarce metal as its amounts in aquatic ecosystems were very low (0.001 to 0.002 mg/l; DWAF 1996). However, natural water may receive Cr from industrial effluents including the corrosion inhibitors and pigments (Galvin 1996) which then become toxic for the aquatic ecosystems. Fish are usually more resistant to Cr than other aquatic organisms, but they can be affected sub-lethally when exposed to the concentrations of 0.013 to 50 mg/l and lethally at 3.5 to 280 mg/l (Moore and Ramamoorthy 1984). The Cr levels (0.13 mg/kg DM = 0.026 mg/kg WW) in fish muscles from the KW site (low polluted) were within the safe limits whereas those from the polluted sites the Cr levels (1.12–1.76 mg/kg DM = 0.23–0.36 mg/kg WW) were higher than 0.05–0.15 mg/kg WW for the food fish (WHO 1985; FEPA 2003). It appeared that the enhanced metal levels in fish organs arise through their bio-magnification at each trophic level. As *L. rohita* is a periphytonic fish, it might have accumulated high Cr levels from the river sediments and phytoplankton. Therefore, it could be inferred that consumption of this fish could cause health hazards in human beings.

Irwandi and Farida (2009) and Rejomon et al. (2009) reported that fish species had a great capacity to accumulate metals, with highest bioaccumulation for essential elements and lowest bioaccumulation for the non-essential elements which is supported by this study. The high metal accumulation in the gills than muscles should be expected as the gills have an intimate contact with their environment. The gills can effectively regulate ions and osmosis as these are the uptake site of waterborne ions where metal concentrations increases especially at the beginning of their exposure, before the metal enters other parts of an organism (Jezierska and Witeska 2001; Jayakumar and Paul 2006). Conversely, the skin which also comes in direct contact with metals showed a far less accumulation as it was evident by this and other studies of Adhikari et al. (2009). The lower metal contents in muscles could be due to their less metabolic activities as reported by Adhikari et al. (2009). As muscles were not active for metal detoxification no metal transport from other organs such as liver and kidney to muscle occurred. The liver of this study appeared to be the second target organ after gills where metal concentrations were higher than muscles and skin. Higher metal concentrations in liver may be attributed to their affinity with metallothionein protein (Ikem et al. 2003) where liver perhaps accumulated metals via blood from other parts of the body including gills and muscles (Kent 1998).

Protein fractions in gills and muscles of fish

The nutritional value of different fish organs depends on their protein, amino acids, vitamins and mineral contents. In the present study, water

pollution appeared to have caused variations in the patterns of protein fractions in the gills and muscle of *L. rohita.* The absence of four major proteins from the gills could be due to either changes in the protein synthetic pathways or the depletion of reserve proteins to overcome the stress of metal pollution as gills are the most exposed organs to the environmental stress. The four new proteins in the fish muscles may be termed as stress proteins which appeared due to the toxic effect of metal pollution of the Indus River (Muthukumaravel et al. 2007). Manna and Mukherjee (1986) reported similar findings in tilapia on exposure to radiation, malathion, and mercuric chloride. Proteins are primarily responsible for cellular responses to physical stimuli, as well as the phenotype of the organism (Barrett et al. 2005; Benninghoff 2007). Also, genes do not code for the post translational protein modifications, the proteolysis and the protein recycling or their sequestration which are also vital for the protein functions (Pandey and Mann 2000; Barrett et al. 2005). The protein changes detected in this study represent a broad range of biological responses, and thus can be used as potential biomarkers in similar studies. Since the gills appeared to be the prime organs for metal toxicity and accumulation, it could be possible that many protein fractions were degenerated under stressful conditions of this aquatic environment (Muthukumaravel et al. 2007). The presence of different patterns of proteins in the gills and muscles of *L. rohita*, confirmed that protein depletion and synthesis could occur in response to the changing environment of *L. rohita* due to the metal pollution. Therefore, all possible measures must be adopted to reduce the pollution in natural water systems to protect aquatic life and the relevant consumers.

DNA profiles of gills and muscles of fish

DNA fragmentation represents an integrative response to the impact of multiple toxic and environmental factors. However, this response is dependent on both the toxicant concentration and exposure duration. It has been demonstrated that pollutant exposure does lead to corresponding increases in DNA damage (Steinert 1999). Any changes to DNA may have long-lasting effects but the self-repairing capability of DNA may affect the precise interpretation of the relevant bioassays (Connell et al. 1999). According to Black et al. (1996), the presence of high molecular weight DNA may not necessarily be a result of the exposure to low impact areas but rather, due to the induction of DNA repair mechanisms after the exposure to a highly impacted area. In fact, many authors have demonstrated that toxicant burdens or exposure duration must reach a threshold level before such repair systems can be initiated (Pavlica et al. 2001; Hoff et al. 2003). The present study showed that the fish muscles at the low, alongside medium and high polluted sites of the Indus River had high molecular weight intact DNA as compared to the DNA in fish gills from the same sites of the Indus River. This suggests that as muscles are the sites of low metabolic activity, their DNA remained intact and no degradation was observed which was also evident from the low metal concentrations in muscles than the gills. Conversely, the low molecular weight DNA observed in the fish gills from the high and medium polluted sites (CH and SK) but not those from the low polluted site (KW) suggested that the fish could be exposed to genotoxicant concentrations that were lower than the threshold levels to initiate the DNA repair mechanism. A few studies have utilized DNA damage as a biomarker to predict the effects at higher levels of biological organization. This study showed parallel responses between the DNA damage and protein fractions in the fish tissues. These changes suggests that the use of DNA damage represented by the relative mobility of low MW DNA fragments and Rf value could be used as a currency to extrapolate relevant sub-cellular effects at higher levels of organization. It is important to consider that an increase in the Rf value is indicative of low molecular weight fragmented DNA whereas, decrease in Rf value and increase in molecular weight reflected the intact DNA. The increase in DNA damage can be explained by an increase in oxidative stress (Almeida et al. 2005) due to perhaps the higher metal levels especially in gills. This might be linked with the contamination of the Indus River water with multiple wastes from the adjacent areas.

Conclusions

These studies confirmed the presence of sublethal concentration of pollutants in Indus River which implied that the fish population was surviving under severe stressful conditions. This statement can be substantiated by the observations of heavy metal load in the fish and the changes in the protein profiles and DNA integrity in the fish gills of this study. The high metal levels in the fish tissues and especially in the edible parts of fish of this study area would have detrimental effects on the health of rural community of Mianwali who heavily depend on fish as a source of food and economy. Therefore, a very close monitoring of metal loads in the Indus River is recommended to minimize the possible risks to the health and livelihood of the consumers. Although no case of metal toxicity due to the fish consumption in this study area has been reported, this may happen in the future if the uncontrolled dumping of the domestic and municipal wastes and less organized agriculture practices are continued in this area. Therefore, it is important to protect the Indus River from the anthropogenic sources of pollution to reduce the environmental risks for the health of the fish and neighboring communities of this and other similar areas.

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