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Environmental Science and Pollution Research

ISSN 0944-1344

Environ Sci Pollut Res
DOI 10.1007/s11356-016-6711-4

Springer

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RESEARCH ARTICLE

First report on fish cysteine as a biomarker of contamination in the River Chenab, Pakistan

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Received: 16 May 2015 / Accepted: 17 April 2016

Abstract The eastern and southern parts of the Faisalabad city produce considerable quantities of industrial and municipal pollutants, much of which is drained into the River Chenab, reducing the productivity of fauna and flora in the river. This study was aimed to determine whether cysteine is useful as a biomarker of exposure to polluted fresh water. The amino acid profile of fish muscle was analyzed by paper chromatography in *Cirrhinus mrigala* and *Labeo rohita* from the River Chenab to determine habitat related variations due to the pollution from industrial and domestic sources. *C. mrigala* showed higher level of metal contamination in muscle tissues for Sn, Cr, Pb, Zn, Mn, Cu, and Cd when compared to *L. rohita*. Both fish species collected from polluted areas of the river Chenab showed significantly ($P < 0.01$) higher levels of metals in comparison to upstream and farmed fish. Farmed *C. mrigala* showed cysteine concentrations in the muscle tissue as 22 ± 1 mg/g dry weight, but concentrations increased to 45 ± 2 mg/g dry weight for fish from a mildly polluted section of the river, and further increased to 83 ± 2 mg/g dry weight in more heavily polluted sections. Cysteine concentration in farmed *L. rohita* was detected as 28 ± 2 and 25 ± 4 mg/g dry weight, respectively for farmed fish and fish from a mildly polluted section of the river, and then increased to 94 ± 3 mg/g dry weight for fish from highly polluted water. *C. mrigala* from a mildly polluted area of the river also had higher levels of cysteine in the muscle, along with increases in aspartic acid, glutamic acid, and alanine. Elevated concentrations of cysteine seem to be associated with a threat to these fish species in polluted sections of the river, and thus may be used as a biomarker.

Keywords Habitat · Heavy metals · Major carps · Amino acids

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-016-6711-4) contains supplementary material, which is available to authorized users.

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Introduction

Heavy metals entering into the environment originate either from anthropogenic sources such as the development of heavy industry or the burning of industrial and domestic waste, and from natural sources such as wind born soil particles. Man-made emissions of elements such as Zn, Ni, Cu, Cd, and Pb greatly exceed the biogenic inputs of these elements (Nriagu 1989). Researchers have tried to discover the informal link between these chemical contaminations of aquatic environments and acute toxicity and genotoxicity of aquatic flora and fauna, especially fish populations (Sharaf et al. 2006; Bolognesi and Cirillo 2014). Some very toxic chemicals that are released into lakes, streams, and rivers, e.g., compounds of Mercury, Zinc, Lead, and Copper, are known to cause mortality in aquatic populations, even at very low concentrations and consumption of contaminated fish can induce genotoxic damage such as chromosomal alterations in the consumer (El-Shehawi et al. 2007; Al-Sabti and Metcalfe 1995). Fish are excellent subjects for the study of the effects of contaminants present in water bodies since they can collect,

Published online: 27 April 2016

metabolize, concentrate, and store water-born pollutants. Biomarkers can be used to elucidate cause-effect and dose-effect relationships in health risk assessment, in clinical diagnosis, and for monitoring purposes (Lam and Gray 2003; Ameur et al. 2012; Bolognesi and Cirillo 2014).

The variations in a range of amino acids upon exposure to different levels of heavy metal pollution were measured in two fish species (*Cirrhinus mrigala* and *Labeo rohita*) collected from upstream and downstream in the River Chenab (a polluted freshwater body) as well as from one pollution-free site (commercial fish farm). These fish species were chosen since they inhabit different niches in fresh water. *C. mrigala* is a bottom feeder and can be used as a bioindicator of sediment pollution. In contrast, because *L. rohita* is a column feeder, it could serve as a bioindicator of contamination of the water column. These fish are also the most economically important freshwater fish species in Bangladesh, India, and Pakistan and are present in most rivers, canals, and streams, as well as being widely reared for commercial purposes (Hussain et al. 2011).

In this study, we analyzed whether the changes in amino acid profile and could serve as a useful biomarker to monitor heavy metal exposure in freshwater fish. The study was therefore designed to provide baseline data on pollution related variability of important amino acids and their increasing or decreasing concentrations according to their habitat pollution load. In this regard, we paid particular attention to cysteine since it has been proposed as a biomarker for air pollution monitoring in some plants (Carfagna et al. 2011). Cobbett and Goldsbrough (2002) have shown that the thiol group present in cysteine makes tight complexes with heavy metals in plants and upon exposure to heavy metals, phytochelatins, such as GSH, required for detoxification are synthesized (Cobbett and Goldsbrough 2002). The use of cysteine as a biomarker for heavy metal pollution in fresh water species has never been tested before. This study was aimed to determine whether cysteine is useful as a biomarker of exposure to polluted fresh water.

Materials and methods

Study area

The Chenab River is a major river in India and Pakistan. It forms in the upper Himalayas in the Lahaul and Spiti district of Himachal Pradesh, India, and flows through the Jammu region of Jammu and Kashmir into the plains of the Punjab, Pakistan. "The total length of the Chenab is approximately 960 kilometers (http://en.wikipedia.org/wiki/Chenab_River). Higher levels of water pollution in the River Chenab have led to a decrease in the population of different species of fish, as well as some extinct, across almost 190 km of its length (Qadir et al. 2007). Industrial and sewage waste from the city of Faisalabad are disposed into the River Chenab, with the river receiving a vast amount of this industrial and municipal waste from the eastern and southern parts of the city through the Chakbandi drain. This highly polluted water contains large amounts of toxic chemicals from a variety of industries such as textiles, chemical, pharmaceutical industries, tanneries, and sugar mills. The level of pollution is quite sufficient to reduce the water productivity of the River Chenab by changing the water quality parameters necessary for the growth of aquatic flora and fauna" (Qadir et al. 2007). This detrimental change in water quality has reduced the population of many fish species, including the major Indian carp.

Water analyses

Water samples were taken from sections of the river at the points of fish harvest and analyzed for selected metals and other water quality parameters defined by the Environmental Protection Agency of Pakistan (2015). In order to maintain calculation standards, seven surface, seven columns, and seven bottom water samples of about 1.5 L each were collected in polypropylene bottles from each site. These were preserved by adding 5 ml of 55 % HNO_3 (Merck, Darmstadt, Germany) to prevent metal adsorption on the inner surface of the container and stored at 4 °C before analysis (Boyd 1981). Each water sample was analyzed separately and endpoint averages were calculated. Metals analyzed were tin (Sn), chromium (Cr), lead (Pb), zinc (Zn), manganese (Mn), copper (Cu), cadmium (Cd), and mercury (Hg). The concentration of each metal was determined using metal kits (Spectroquant® Analysis System, Merck) and using nitrous oxide/acetylene flame atomic absorption spectrophotometry (2000 series, High-Technologies Corporation, Chiyoda, Tokyo, Japan) with Zeeman background correction. The blanks and calibration standard solution were also analyzed in the same way as for the samples. The instrument calibration standards were set by diluting standard of these metals (1000 ppm) supplied by Merck, Germany. A known 1000-mg/L concentration of all the above-mentioned metal solutions were prepared from their salts. All chemicals and reagents used were of analytical grade (Merck, Darmstadt, Germany).

Procurement of fish

Fish were procured from three different sites based reported information by Qadir (2008), which were designated, respectively, as "highly polluted section of the river", "mildly polluted section (upstream to the highly polluted section)" and "farmed fish" as a control. Farmed fish were procured from a fish seed hatchery at Satiana Road, Faisalabad, Pakistan. *L. rohita* (800–1200 g) and *C. mirgala* (800–1250 g) were collected using gill nets, drag nets, and pocket nets along the length of the River Chenab downstream of the entrance to the

Chakbandi Drain at 31.570° latitude and 72.534° longitude. Fifteen fishes of each fish species was collected from each sampling site and hence total 45 fishes of each species were collected from three sampling sites. Each sampling site divided into three substations. The samples of each fish species from each sampling sites were processed. Maximum total length of fish measured from the tip of the snout to the end of the compressed caudal fin was used (Abowei 2010) to measure the Fulton's condition factor.

Preparation of fish samples

All the fish specimens were collected and transported in polyethylene bags to the Fisheries Laboratory of GC University, Faisalabad, Pakistan. The muscle tissues of the fish samples were excised, washed with distilled water, and cut into small pieces (2–3 cm) with a knife. Then, the tissue was oven dried at 65 °C for 24 h, powdered using a glass mortar, sieved through 1 mm mesh, and stored in airtight plastic vials kept in desiccators.

Metal analysis

Five specimens from each species were used for heavy metal analysis. The tissues were oven dried at 70 to 73 °C until a constant weight was obtained. The specimens were then ground to a fine powder and stored in desiccators in order to avoid moisture accumulation before digestion. The digestion procedure was carried out as described by Kotze et al. (2006). The concentration of each metal was determined using heavy metal kits (Spectroquant® Analysis System, Merck).

Amino acid analysis

The amino acid profile of the fish muscle tissues was determined by paper chromatography in the following phases.

Hydrolysis

Weighed samples of finely crushed dried fish meat were sealed with 6 M HCl in a glass ampoule and heated at 110 °C for 24 h. This hydrolysis broke the peptide bonds between the amino acids, leaving free amino acids in the ampoule. The ampoule tube was then broken and the solvent was evaporated to leave the constituent amino acids.

Paper chromatography

A chromatography sheet (Whatman Chromatography paper (21 × 21 cm)) spotted with the samples by capillary tube was developed vertically irrigated for 18–20 h in n-butanol: acetic acid: water (butanol 40 %, acetic acid 10 %, and water 50 %) solvent system. The paper was then heat dried at 90 °C and sprayed with 0.1 % ninhydrin ethanolic solution. The amino acids were identified by comparing the Rf values of the spots to those of an amino acid standard (Grable et al. 1964).

Paper electrophoresis

Paper electrophoresis was used to further separate spots that were difficult to separate by use of paper chromatography. Spots appearing so close together on the chromatography paper as to be difficult to distinguish were irrigated in a thin streak along the line of origin with a phosphate buffer (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 dissolved in 800 mL of distilled H_2O, with pH adjusted to 7.0 using HCl and with the volume made up to 1 L by adding H_2O). A charge of 1.5 KV was passed for 1 h and the paper was dried for 15 min at 70 °C. Then, this paper was sprayed with 0.1 % alcohol-ninhydrin solution and again heated at 70 °C for 10 min. After drying, amino acids were confirmed by comparing the purple spots with amino acid standards (Grable et al. 1964).

Quantitative estimation

After paper chromatography, the colored spots of amino acids were cut with a scissor and eluted with 3 mL methanol. The optical density of the elute was determined at 550 nm and quantitative results were obtained by comparison to a standard curve for the respective amino acids (Grable et al. 1964).

Statistical analysis

Data were reported as mean ± standard error. One-way ANOVA and the Tukey's multiple comparison test were used to highlight the statistically significant differences among the three groups (farmed, highly polluted, and mildly polluted sites). Significance was checked at $P < 0.05$. All statistical analyses were performed using the program SPSS 9 for PC.

Results and discussions

The River Chenab was found profoundly polluted with heavy metals, and all water quality parameters (WQPs) were observed as far beyond the WHO permissible limits. Highly alkaline pH, higher amount of total, dissolved, and suspended solids resulting in higher values of BOD and COD have made this habitat unfavorable for the growth of fish (Tables 1 and 2). Phenols were reported significantly ($P < 0.05$) in higher concentrations enough to induce proximate changes in meat quality. Phenols occurrence in aquatic ecosystems is also related industrial and municipal sewages and released from the degradation of numerous pesticides. Some phenols are formed during natural processes. Phenols were reported as harmful

Table 1 Morphometric measurements of experimental fish species

Variables	Wet weight (g)	Standard Length (cm)	Total length (cm)	Head length (cm)	K
L. rohita					
Fish from site R1[a]	1088 ± 67b	40 ± 1.21a	46 ± 2.61a	5.9 ± 0.80b	1.70
Fish from site R2	1121 ± 51a	45 ± 2.84a	49 ± 1.20a	5.7 ± 0.71b	1.23
Fish from site R3	1099 ± 83b	44 ± 3.14a	51 ± 2.0b	6.1 ± 0.92b	1.29
Fish from upstream	1141 ± 91c	48 ± 6.75b	55 ± 3.24c	6.8 ± 1.17a	1.03
Farmed fish	997 ± 22d	38 ± 1.90a	43 ± 2.22a	4.9 ± 0.87c	1.91
C. mrigala					
Fish from site R1	1016 ± 82b	48 ± 3.29a	54 ± 2.51c	5.4 ± 0.79b	0.91
Fish from site R2	1074 ± 99b	46 ± 2.95a	51 ± 2.01b	4.9 ± 0.82b	1.10
Fish from site R3	1199 ± 72bc	51 ± 3.64c	58 ± 2.37c	6.7 ± 0.93a	0.90
Fish from upstream	1065 ± 96b	49 ± 6.75b	57 ± 1.01c	5.6 ± 0.98b	0.92
Farmed fish	982 ± 16d	41 ± 2.91a	48 ± 1.11a	5.2 ± 0.94b	1.43

Values (mean ± SE) are average of fifteen fish samples; means sharing similar letters in a column are statistically non-significant ($P > 0.05$)

K Fulton's condition factor

[a] R1–R3: fish from polluted sites of the River Chenab

ecotoxins having genotoxic, mutagenic, hematotoxic, and carcinogenic nature to both fish and human (Pulkrabová et al. 2007; Havelková et al. 2008; Dû-Lacoste et al. 2012). Compared to the control, a significant amount of heavy metals ($P < 0.01$) was determined in the muscle tissues of the fish tested from the highly/mildly polluted sites (Fig. 1), indicating significant bioaccumulation, particularly in *C. mrigala*. Present findings of elevated levels of heavy metal

Table 2 Water quality parameters of the River Chenab

Water quality parameters	Highly polluted habitat[a]	Mildly polluted (upstream) habitat[b]	Farmed (control)
pH	11.61 ± 2.22a	8.10 ± 0.75b	7.90 ± 1.02b
BOD (mg L^{-1})	76.64 ± 3.21a	44.95 ± 2.25b	33.52 ± 3.10c
COD (mg L^{-1})	195.45 ± 5.52a	67.22 ± 3.66b	60.04 ± 4.11b
TDS (mg L^{-1})	2399.57 ± 22.47a	1295.71 ± 8.99b	319.42 ± 8.47c
TSS (mg L^{-1})	3088.34 ± 4.13a	1760.55 ± 5.59b	179.31 ± 6.82c
Salinity (mg L^{-1})	1942.66 ± 20.21a	401.72 ± 8.21b	200.22 ± 5.32b
Conductivity (mS m^{-1})	3.22 ± 0.76a	1.32 ± 0.23b	0.19 ± 0.01c
Phenols (mg L^{-1})	2.22 ± 0.81a	0.72 ± 0.01b	0.14 ± 0.00c
Sulfates (mg L^{-1})	431.54 ± 5.71a	81.42 ± 1.58b	59.12 ± 6.55b
Trace metal contamination (mg L^{-1})			
Tin	0.42 ± 0.02a	0.001 ± 0.00b	0.001 ± 0.00b
Chromium	0.48 ± 0.01a	0.28 ± 0.02b	0.03 ± 0.00c
Lead	2.02 ± 0.01a	0.14 ± 0.00b	0.09 ± 0.00c
Zinc	0.34 ± 0.01a	0.18 ± 0.01b	0.029 ± 0.01c
Manganese	2.03 ± 0.04a	1.86 ± 0.01a	0.36 ± 0.03b
Copper	1.62 ± 0.03a	0.93 ± 0.06b	0.041 ± 0.02c
Cadmium	0.10 ± 0.01a	0.11 ± 0.02a	0.003 ± 0.01b
Mercury	0.94 ± 0.01a	0.008 ± 0.01b	<0.001

Values (mean ± SE) are average of five water samples analyzed in triplicate. Means sharing similar letters in a row are statistically non-significant ($P > 0.05$)

BOD biological oxygen demand, *COD* chemical oxygen demand, *TDS* total dissolved solids, TSS: Total suspended solids

[a] R1–R3: water from polluted sites of the river Chenab

[b] Upstream to polluted river sites

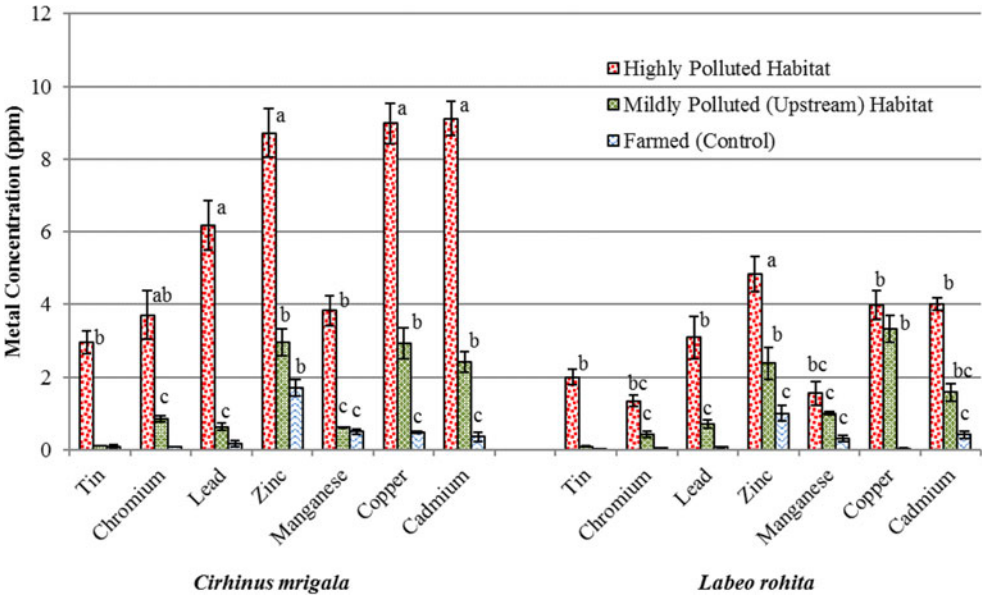

Fig. 1 Heavy metal concentrations in the muscle tissues of *C. mrigala* and *L. rohita* collected from three different environments. *Different letters* (*a*, *b*, *c*) indicate statistically significant differences ($P < 0.01$)

concentrations severely affect the health and normal physiological functions of fish corroborating the findings of Swann (1997). Heavy metal pollution also creates severe problems by injuring the biological functions in aquatic organisms and their accumulation in fish tissues, specifically in meat lead to serious health hazard to the consumers (Daoud et al. 1999). The Fulton's condition factor (K) for indication of fish health of both species is indicated in Table 1 along with other morpho-metric measurements. Upstream area fish species showed comparatively less value indicating the significance of the comparison. *L. rohita* showed higher values for K probably indicating sensitivity for pollution when compared to *C. mrigala* (Nehemia et al. 2012).

The amino acid profile of the fish clearly demonstrated that some of the important amino acids showed considerable variations related to their environment. Among essential amino acids valine, leucine, isoleucine, phenylalanine, methionine, threonine, lysine, arginine, and histidine were recorded in variable concentration in *C. mrigala*. Interestingly, methionine, phenylalnine, and histidine were not detected in *C. mrigala* collected from polluted site of the river (Fig. 4). Cysteine, tyrosine, aspartic acid, serine, glycine, proline, glutamic acid, and alanine were detected in *C. mrigala* as non-essential amino acids. The differences remained non-significant for the fish collected from polluted and non-polluted sites for tyrosine, serine, glycine, and glutamic acid. Essential amino acids valine, leucine, isoleucine, phenylalanine, methionine, threonine, lysine, arginine, and histidine were recorded with different concentration in *L. rohita* collected from control, polluted, and upstream sites. However, phenylalanine and arginine were not recorded in *L. rohita* harvested from polluted site of the river (Fig. 5). Cysteine, tyrosine, aspartic acid, serine,

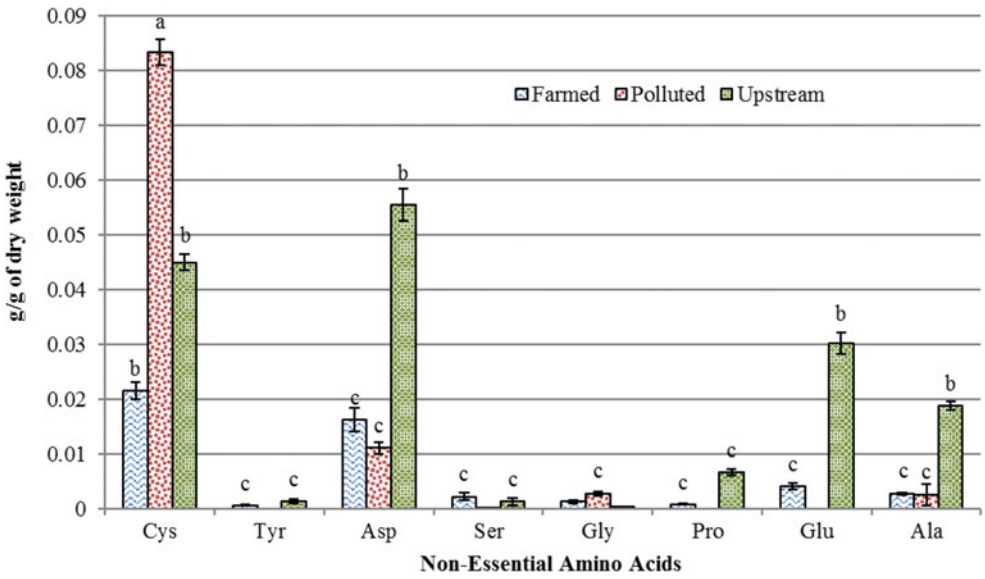

Fig. 2 Non-essential amino acids in the muscle tissues of *C. mrigala* collected from three environments. *Different letters* (*a*, *b*, *c*) indicate statistically significant differences ($P < 0.01$)

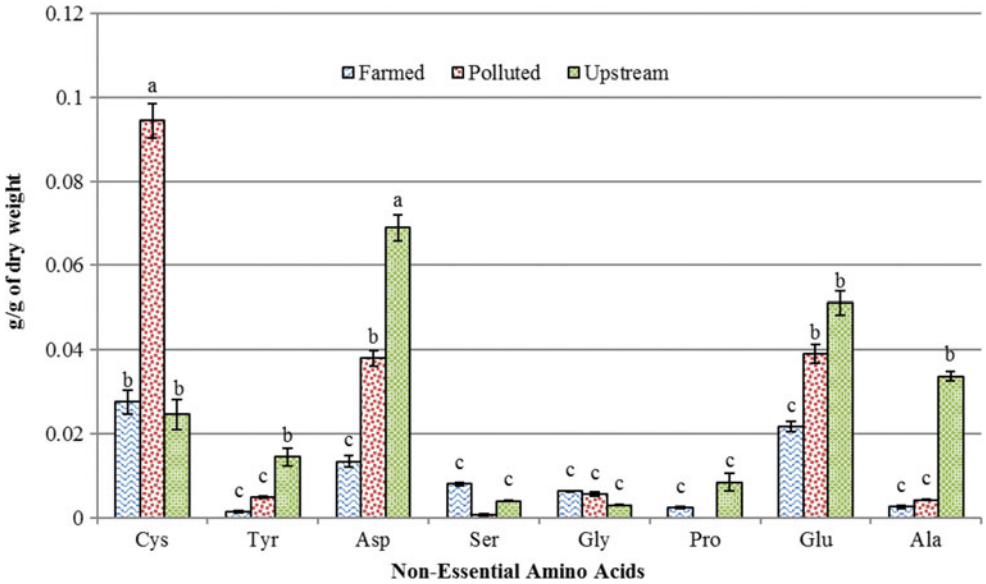

Fig. 3 Non-essential amino acids in the muscle tissues of *L. rohita* collected from three environments. *Different letters* (*a*, *b*, *c*) indicate statistically significant differences ($P < 0.01$)

glycine, proline, glutamic acid, and alanine were detected in *L. rohita* as non-essential amino acids. Proline was not detected in *L. rohita* harvested from polluted site of the river (Fig. 3). No statistical differences were found for cysteine in *L. rohita* from farmed and mildly polluted river section (Fig. 6). Among non-essential amino acids, cysteine showed a considerable increase in its concentration in both fish species collected from the highly polluted environment (Fig. 2). In the case of *C. mrigala*, samples collected upstream from the highly polluted area also showed an increase in the concentration of cysteine, perhaps because its bottom feeding nature renders it susceptible to increased exposure to pollution. No variation in cysteine concentration was observed in the case of *L. rohita* collected from the upstream areas, however (Fig. 3). Methionine was not detected in the muscles of *C. mrigala* collected from the highly polluted area (Fig. 4) although a reasonable amount was found in the muscles of *L. rohita* (Fig. 5). Similar variations were observed for aspartic acid, glutamic acid, and alanine in both fish species. Although changes were evident in other amino acids, the changes in cysteine content in fish collected from the highly polluted environment were clearly the most highly significant ($P < 0.01$) response to pollution (Fig. 6), and suggest that cysteine represents a good candidate as a biomarker for pollution in fish. Few other studies also substantiate our findings, a study by Arafa and Ali (2008) reveled that heavy metal pollution also decreases the intestinal absorption of amino acids leading to the decline in musculature levels of fishes. Another study by Neff (1985) also reported that animals under toxic stress, diversification of energy occurs to accomplish the impending energy demands leading to the protein level depletion. Proteins are highly sensitive to heavy metal poisoning

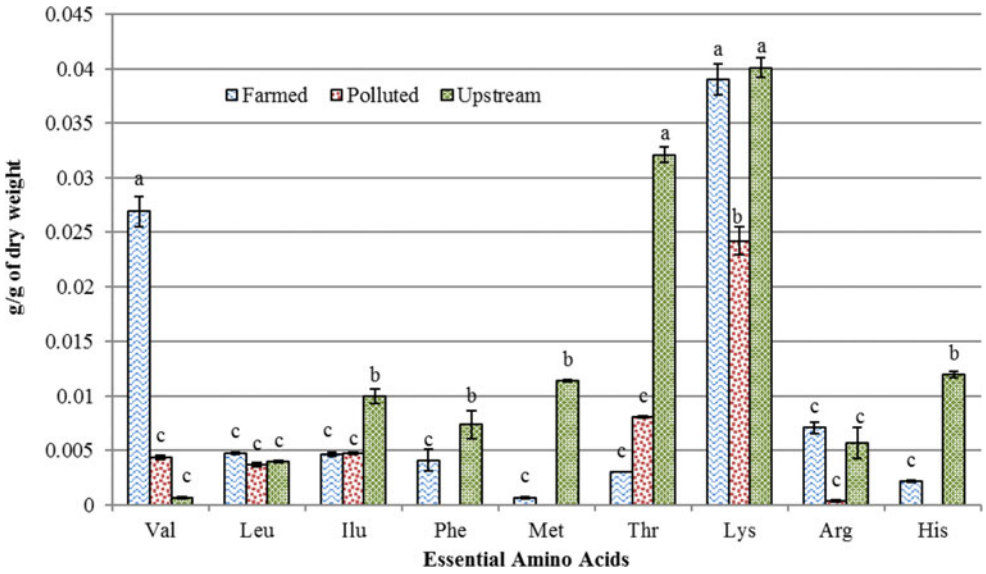

Fig. 4 Essential amino acids in the muscle tissues of *C. mrigala* collected from three environments. *Different letters* (*a*, *b*, *c*) indicate statistically significant differences ($P < 0.01$)

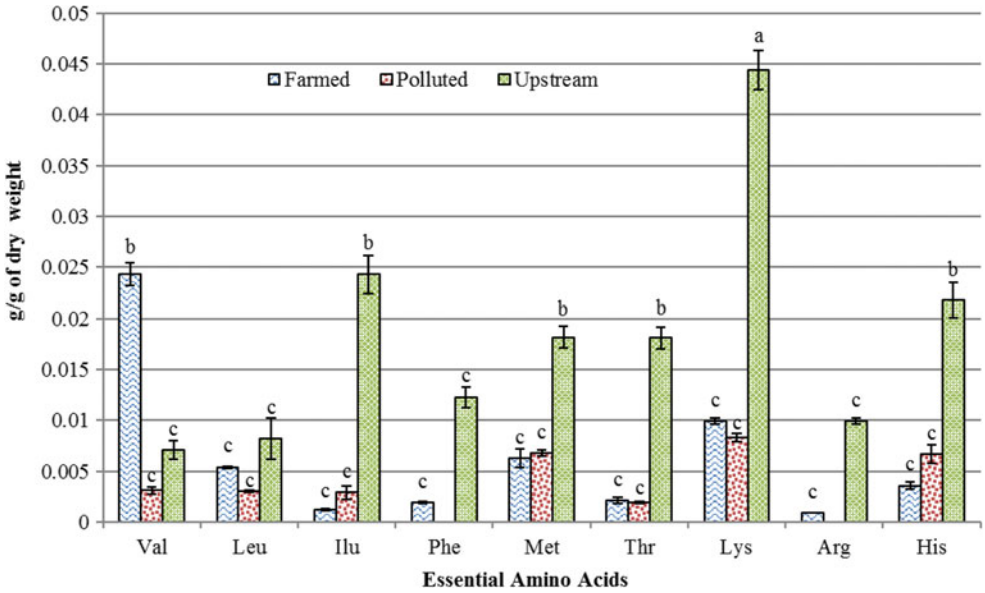

Fig. 5 Essential amino acids in the muscle tissues of *L. rohita* collected from three environments. *Different letters* (*a*, *b*, *c*) indicate statistically significant differences ($P < 0.01$)

(Jacobs et al. 1977). Some biochemical studies in the literature clearly demonstrated that concentration of protein in *Oreochromis niloticus* was significantly decreased compared with the fish of the reference aquaculture in respect of heavy metal pollution (Arafa and Ali 2008). The present findings are also in agreement of Sobha et al. (2007) who found a decline in the musculature protein content on exposure of the freshwater fish *Catla catla* to cadmium.

Pollution associated increase in cysteine and its biological effects on fish can be understood in the context of its relationship with metallothioneins and methionine. Metallothioneins (MTs) are cysteine-rich and heat-stable proteins that bind to metals through metal–thiolate bonds (Kaegi and Schaeffer 1988). MTs have three major physiological roles: (1) detoxification of certain trace metals (Goering and Klaassen 1984), (2) internal homeostasis of Cu and Zn (Brouwer et al. 1986), and (3) participation in metabolic functions (Roesijadi et al. 1998). Several isoforms and different metals bind to these proteins exist in different fish organs (Vasak 2005). Such cysteine-rich proteins, forming a distinctive set of protein frameworks and folds, are found in all living organisms and often play crucial roles as growth factors, hormones, ion channel modulators, and enzyme inhibitors in various biological pathways (Lavergne et al. 2012).

Metal pollution, therefore, may provoke an increased demand for cysteine so as to support the synthesis of MTs but this cysteine demand is unlikely to be met from the diet. As a nonessential amino acid, however, cysteine can be synthesized from its essential amino acid precursor, methionine (National Research Council 2011). In this regard, it is

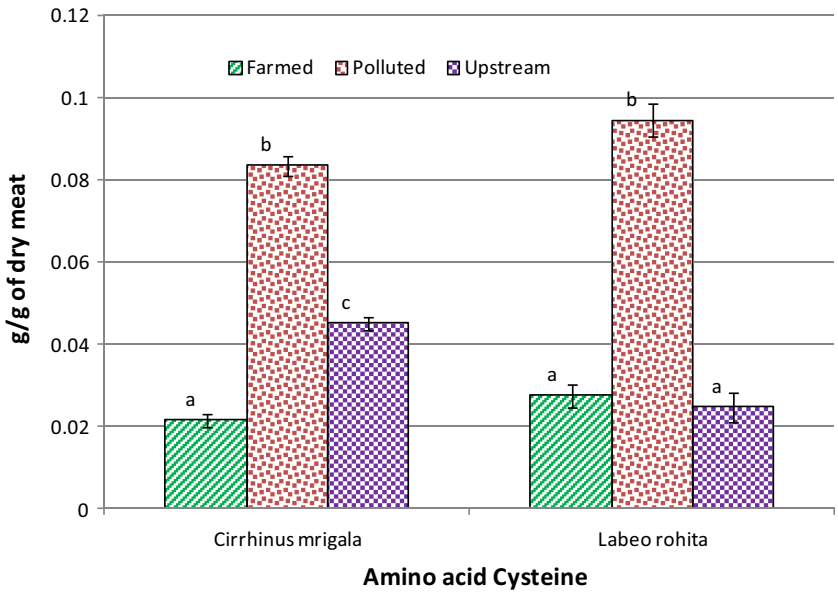

Fig. 6 Concentration of cysteine in the fish muscle tissues from three different environments. *Different letters* (*a*, *b*, *c*) indicate statistically significant differences ($P < 0.01$)

noteworthy that this study also shows that the concentration of methionine was reduced in fish from the polluted area. Arafa and Ali (2008) obviously found that the levels of the amino acids, methionine, lysine, and cystine in fish from heavy metal pollution load were significantly decreased in comparison to reference fish. Studies by Ansari (1986) showed that the exposure of *Channa punctatus* fish to 1 ppm of copper sulfate for 84 days led to disappearance of cystine from the musculature supporting our view of methionine conversion to cysteine. Another reason of the reduction of methionine may also be demonstrated as heavy metals inhibit the intestinal absorption of essential amino acids like lysine and methionine in fishes (Farmanfarmaian et al. 1985). Studies have also confirmed, however, that sufficient levels of methionine are required for optimal growth, feed efficiency, survival, and nutrient utilization, and therefore the reduction in methionine to support cysteine and thence MT synthesis in an attempt to cleanse pollutants has a knock-on effect in terms of retarded growth and increased mortality (Mdar et al. 2012). In this study, the highly significant ($P < 0.01$) increase in the concentration of cysteine may therefore be directly correlated to the reduced population of *L. rohita* and *C. mrigala* in this polluted section of the river (Arafa and Ali 2008; Qadir et al. 2007).

Overall, there is a lack of information available on particular amino acid variability so the findings of the present study could be used as baseline information to expand research in this area in the future. Our results suggest, however, that variability in the level of cysteine may be used as a new biomarker for the screening of environmental contaminants and to assess the health of fish.

Conclusion

Higher concentrations of cysteine are a fish's response to pollution, resulting in a reduction in methionine, an adequate level of which is required for optimal growth, feeding efficiency, nutrient utilization, and survival. These biomolecules could be used for biomarker studies for environmental screening.

Acknowledgments The authors would like to express their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RG-1435-012.

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