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RESEARCH ARTICLE

Diagnosis of Metals Induced DNA Damage in Fish Using Comet Assay

Safina Kousar* and Muhammad Javed

Fisheries Research Farms, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad,*Present Address: GC Women University, Faisalabad, Pakistan
*Corresponding author: safinazoologist@gmail.com

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ABSTRACT

Among xenobiotics, metals gained attention because of their toxicity and harmful effects on aquatic ecosystems, fish and the human health. Present experiment was conducted to determine the genotoxic effects of individual metals viz. arsenic (As), copper (Cu), and zinc (Zn) in peripheral blood erythrocytes of four freshwater fish species viz. *Labeo rohita* (rohu), *Cirrhina mrigala* (mori), *Catla catla* (thaila) and *Ctenopharyngodon idella* (grass carp) by using Comet assay. For this purpose fish were exposed to four different sub-lethal concentrations (17, 25, 33 and 50% of LC_{50}) of As, Cu, and Zn for a period of 30-day. All the four fish species showed significantly different extant of DNA damage in-terms of percent damaged cells, genetic damage index (GDI) and cumulative tail length of comets due to metals exposure. Among four fish species, *Cirrhina mrigala* showed significantly ($P<0.05$) higher percentage of damaged cells, GDI and cumulative tail length of comets with the mean values of 52±6%, 1.6±0.3 and 152±35 µm, respectively. However, *Catla catla* showed significantly lower percentage of damaged cells and GDI with the mean values of 29±4% and 1.1±0.1, respectively. This study indicated that individual metals and metals mixture, existing in aquatic habitats of Pakistan can induce DNA damage in indigenous fish fauna.

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INTRODUCTION

Genetic toxicology deals with the interaction of DNA damaging agents i.e. metals with the cell's genetic material in relation to subsequent effects on organism's health (Theodorakis *et al*., 1998). Metals gained attention because of their toxicity, accumulation and harmful effects on aquatic ecosystems, fish and the human health (Javed, 2012; Mudgal *et al*., 2010; Ilyas and Javed, 2013). Although much work on LC_{50} of various metals for fish was done by different workers (Roy *et al*., 2006; Azmat *et al*., 2012; Kousar and Javed, 2012; 2014) but the genotoxic effects of these metals on various fish systems are yet to be characterized.

Metals can induce DNA damage in different ways. As, present in air, water and soil causes DNA damage in fibroblast cells through different modes of action (Bhattacharya and Bhattacharya, 2007; Naz and Javed, 2013). It can cause cellular transformation (Huang *et al*., 1995), gene amplification (Rossman and Wolosin, 1992), DNA strand breakage and DNA-protein cross-links (Gebel *et al*., 1998). Being a fenton metal, Cu has the ability to contribute in redox cycling, ultimately leading to the production of reactive oxygen species (ROS), such as hydroxyl radical (Valko *et al*., 2005). It has the ability to bind directly to DNA (Moriwaki *et al*., 2008; Javedl, 2013; Shaukat and Javed, 2013). In- vivo zinc exposure may cause single strand breaks which can be measured by means of Comet assay (Banu *et al*., 2001; Auon *et al*., 2014).

Comet assay is used as one of the best approaches to study the genotoxic effects of pollutants on fish (Nagarani *et al*., 2012), as it is used for the estimation of DNA damage to evaluate the genetic risk associated with xenobiotic exposures. It is considered reliable, responsive and fast technique for the detection of DNA single/double strand breakage and alkali-labile sites, induced in individual eukaryotic cells by physical and chemical agents (Kim *et al*., 2002). This assay has effectively been applied to peripheral erythrocytes of various fish species exposed to diverse genetic toxicants (Matsumoto *et al*., 2006). Monitoring of xenobiotics in freshwater ecosystem by means of resident species can help to estimate environmental quality as well as human health.

Determination and localization of the toxicant's concentration ensure the life of animal species that are of importance for the human health (Nicareta, 2004). Pakistan is among the countries facing acute fresh water pollution problems where only 1% of industrial water is treated before its discharge into the rivers and streams (Khan *et al*., 2012). Thus, heavy discharges of untreated water into the rivers of Pakistan is adversely affecting the fresh water fisheries (Jabeen and Javed, 2012) and the indigenous fish species are on the verge of extension in the rivers of Pakistan (Rauf *et al*., 2009). Comparative toxicity studies are needed to identify the effects of metals pollution on the fish and their tolerance limits to devise proper strategies for their conservation in the natural aquatic habitats. Thus, it is imperative to study the responses of selected fish species to metallic ion toxicity that could induce genotoxicity due to accumulation of metals in their bodies.

MATERIALS AND METHODS

The experiments were conducted in the laboratories of Fisheries Research Farms, Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan. Four fish species (*Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella*) of 150-day age were collected from the Fish Seed Hatchery, Faisalabad and transported to the wet laboratory with proper care. Fingerlings of all the four fish species were acclimatized to laboratory conditions for two weeks prior to experiments. During acclimation period fish fingerlings were fed to satiation on feed twice daily. Remains of feed and excretory waste were siphoned off daily to avoid stress on the fish. Analytical grade (Merck) compounds viz. As_2O_3, $CuCl_2.6H_2O$ and $ZnCl_2$. $2H_2O$ were used to make stock solutions of As, Cu and Zin. Stock solutions were further diluted up to desired metal concentrations for use in genotoxicity studies. The 96-hr LC_{50} of each metal for fish was obtained by following Probit analysis procedure as mentioned in Azmat *et al.* (2012). All tests were performed at constant water temperature (30^oC), pH (7.5) and hardness (300 mg/L^{-1}). To determine DNA damage in erythrocytes, each fish species was exposed separately to four different sub-lethal water-borne concentrations (17, 25, 33 and 50% of 96-hr LC_{50}) of As, Cu, and Zn in the aquaria for 30 days. During exposure period, the fish were fed on diet (34% DE and 3 Kcal/g DE) to-satiation twice a day. Fish blood samples were obtained from caudal vein and processed for Comet assay according to Singh *et al.* (1988). After electrophoresis, the slides were neutralized gently with 0.4 M Tris buffer at pH 7.5 and DNA stained with ethidium bromide (20μg/ml). One hundred and fifty cells (50 per replicate) were scored and examined randomly under Epi-Fluorescence microscope equipped with light source of mercury short arc reflector lamp filters for ethidium bromide at 400 X magnification and low lux camera. Cells with no DNA damage possess intact nuclei without a tail, whereas cells with DNA damage showed comet like appearance. The length of DNA migration in the comet tail was estimated as DNA damage (Grover *et al.,* 2003). The cells with no head or dispersed head were regarded as apoptotic cells and were not included in the analyses. The DNA damage was quantified by visual classification of cells into five categories "Comets" corresponding to the tail length, undamaged: Type 0 (Fig. 1a), low-level damage: Type I, medium-level damage: Type II, high-level damage: Type III and complete damage: Type IV (Fig. 1b). Means were compared for statistical differences through Tukey's student Newnan-Keul test by following Computer package. Both parametric and non-parametric tests were used in order to evaluate statistical differences at $P<0.05$.

RESULTS

Table 1 shows significantly variable 96-hr LC_{50} of As, Cu and Zn for *Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella*. Table 2 shows percentage of damaged cells, genetic damage index and cumulative tail length of comets observed in peripheral blood erythrocytes of all the four fish species at four different exposure concentrations of metals along with negative and positive control treatments. All the four fish species showed significantly higher DNA damage, genetic damage index and cumulative tail length of comets at 50% of metals LC_{50} except *Ctenopharyngodon idella* that showed higher of damaged cells, genetic damage index and cumulative tail length of comets due to positive control treatment instead of Cu exposure. All the three exposure metals caused concentration dependent increase in DNA damage in peripheral blood erythrocytes of all the four fish species (Table 2).

Percentage of Damaged Cells (%): Regarding overall performance of four fish species towards induction of DNA damaged cells, under the exposure of metals, all the four fish species showed significantly higher frequency of damaged cells due to exposure of As (Table 3). Among the four fish species, the percentage of damaged cells in fish erythrocytes varied significantly as: *Cirrhina mrigala* > *Labeo rohita* ≥ *Ctenopharyngodon idella* > *Catla catla*. All the four fish species showed significantly ($P<0.05$) higher sensitivity towards As exposure as it caused significantly maximum damage to the DNA (44±9%) while it was significantly lowest (35±0.9%) due to Cu exposure. The abilities of various metals to cause damage in the erythrocytes of four fish species followed the order: As > Cu > Zn (Table 3).

Genetic Damage Index: The exposure of four fish species to metals resulted into significant variability in their GDI values (Table 3). The exposure of As caused significantly higher GDI, closely followed by that caused by Zn and Cu exposures with non-significant difference for *Labeo rohita* only. In case of *Cirrhina mrigala*, the mean GDI was significantly higher due to As exposure with the mean value of 1.8±0.9, followed by that of Zn (1.6±1). In case of *Ctenopharyngodon idella*, As exposure caused significantly maximum damage (1.4±0.7) to their erythrocytes while it was significantly lowest (0.9±0.5) due to Cu. Regarding mean ability of four fish species to induce genetic damage in their erythrocytes, As caused significantly maximum damage (1.4±0.3). *Cirrhina mrigala* appeared significantly more sensitive to exhibit significantly higher mean GDI of 1.6±0.2, followed by

Table 1: Acute toxicity of metals for fish

Metals	Fish species	Mean 96-hr LC_{50} (mgL^{-1})	95% CI (mgL^{-1})	Mean lethal Conc. (mgL^{-1})	95% CI (mgL^{-1})
	Labeo rohita	30.00±0.02a	27.78 - 31.78	40.16±0.04a	37.13 - 46.88
	Cirrhina mrigala	24.50±0.05b	23.17 - 25.53	32.06±0.02b	30.34 - 35.06
As	*Catla catla*	10.16±0.22d	9.42 - 10.70	14.05±0.20d	13.16 - 15.69
	Ctenopharyngodon idella	22.17±0.02c	20.82 - 23.20	29.67±0.02c	28.03 - 32.49
	Labeo rohita	33.41±0.03a	31.19 - 35.18	45.53±0.04a	42.76 - 50.28
	Cirrhina mrigala	17.17±0.24c	15.88 - 18.17	24.33±0.12c	22.73 - 27.11
Cu	*Catla catla*	16.85±0.20d	15.64 - 17.83	23.83±0.05d	22.24 - 26.55
	Ctenopharyngodon idella	21.36±0.42b	19.72 - 22.61	31.61±0.26b	29.49 - 35.19
	Labeo rohita	165.29±0.44a	154.74 - 173.25	212.87±0.89a	200.89 - 235.14
	Cirrhina mrigala	108.77±0.07b	102.13 - 113.60	140.69±0.12b	132.66 - 156.45
Zn	*Catla catla*	41.81±0.26d	39.08 - 43.92	57.29±0.56d	53.84 - 63.30
	Ctenopharyngodon idella	99.57±0.09c	93.92 - 104.08	129.22±0.08c	122.00 - 142.20

CI = Confidence Interval; Means with similar letters in a single column (for each age group) are statistically non-significant at p<0.05.

Table 2: DNA damage in peripheral erythrocytes of *Labeo rohita, Cirrhina mrigala, Catla catla* and *Ctenopharyngodon idella* due to metals exposure

Species/ Parameters	Exposure Metals											
	As				Cu				Zn			
	Exposure concentrations ($mg\ L^{-1}$)	% age of damaged cells (II+III+IV)	Genetic damage index * (GDI)	Cumulative tail length (µm)	Exposure concentrations ($mg\ L^{-1}$)	% age of damaged cells (II+III+IV)	Genetic damage index * (GDI)	Cumulative tail length (µm)	Exposure concentrations ($mg\ L^{-1}$)	% age of damaged cells (II+III+IV)	Genetic damage index *(GDI)	Cumulative tail length (µm)
Negative control	0.00	2±2.00f	0.1±0e	3±0.40f	0.00	0.7±1f	0.1±0f	3±0.4f	0.00	0.7±1f	0.1±0.0f	4±0.13f
Positive control	CP (20 µgg-1)	44±3.46d	1.7±0.1b	118±0.18c	CP (20 µgg-1)	40±1c	1.4±0c	114±0.2c	CP (20 µgg-1)	48±4c	1.6±0.1c	118±0.29c
17% of LC_{50}	5.10	32±5.46e	1.0±2d	78±0.44e	5.68	18±0e	0.7±0e	52±0.1e	28.10	5±2e	0.6±0.0e	28±0.15e
25% of LC_{50}	7.50	51±1.15c	1.5±0.1c	112±0.65d	8.35	36±1d	1.2±0d	78±0.1d	41.32	26±1d	0.9±0.0d	68±0.18d
33% of LC_{50}	9.90	60±4.16b	1.7±0.1b	167±0.22b	11.03	60±2b	1.8±0b	174±1.0b	54.55	74±1b	2.1±0.0b	143±0.29b
50% of LC_{50}	15.00	76±3.46a	2.2±0.2a	195±0.0a	16.71	66±3a	2±0.1a	205±0.4a	82.65	78±1a	2.7±0.0a	222±1.11a
Negative control	0.00	1±1f	0.06±0d	4±0.2f	0.00	0±0.00f	0.04±0f	3±0.2f	0.00	2±2f	0.09±0f	3±0f
Positive control	CP (20 µgg-1)	51±4d	1.6±0c	121±0.1e	CP (20 µgg-1)	46±0.00d	1.5±0d	119±0.2d	CP (20 µgg-1)	45±5d	1.4±0d	122±0d
17% of LC_{50}	4.17	48±4e	1.7±1c	139±0.1d	2.92	20±3.15e	1±1e	91±0.3e	18.49	13±3e	0.6±0e	49±0e
25% of LC_{50}	6.13	76±2c	2.3±0b	240±0.2c	4.29	58±0.00c	1.8±0c	136±0.2c	27.19	82±1c	2.3±0c	156±0c
33% of LC_{50}	8.09	82±2b	2.3±0b	307±0.2b	5.67	68±2.00b	2±0b	177±0.2b	35.89	90±2b	2.4±0b	190±0b
50% of LC_{50}	12.25	86±1a	2.7±0a	347±0.1a	8.59	78±2.00a	2.4±0a	238±0.4a	54.39	92±2a	2.7±0a	298±1a
Negative control	0.00	0.6±1f	0.1±0.0e	3±0.2f	0.00	1±1f	0.1±0e	3±0.1f	0.00	1±1.1e	0.1±0.0c	3±0.f
Positive control	CP (20 µgg-1)	45±2c	1.6±0.0b	120±0.8c	CP (20 µgg-1)	48±3c	1.8±0.9b	122±0.5c	CP (20 µgg-1)	44±1.1a	1.6±0.0a	110±0.2b
17% of LC_{50}	1.73	12±4e	0.5±0.1d	52±0.1e	2.86	4±0e	0.4±0.0d	20±0.1e	7.11	10±3.4d	0.5±0.0d	47±0.2e
25% of LC_{50}	2.54	24±5d	0.9±0.1c	107±0.0d	4.21	18±2d	0.8±0.1c	86±0.5d	10.45	17±3.0c	0.7±0.0c	87±0.9d
33% of LC_{50}	3.35	48±5b	1.7±0.1b	148±0.2b	5.56	56±4b	1.9±0.1b	136±0.2b	13.80	30±0. b	1.0±0.0b	98±0.3c
50% of LC_{50}	5.08	70±3a	2.2±0.0a	190±0a	8.43	68±2a	2.2±0.1a	176±0.4a	20.91	42±0.0a	1.4±0.0a	112±0.5a
Negative control	0.00	2±2f	0.1±0.0e	3±0e	0.00	2±1f	0.1±0.0f	3±0.3f	0.00	2±1.1f	0.1±0.0d	3±0.1f
Positive control	CP (20 µgg-1)	55±1b	1.7±0.0b	109±0c	CP (20 µgg-1)	54±4a	1.6±0.1 a	115±0.3a	CP (20 µgg-1)	58±2.0c	1.7±0.0ab	119±0.9b
17% of LC_{50}	3.77	31±1e	1.3±0.0d	90±0d	3.63	18±1e	0.6±0.0e	33±0.5e	16.93	14±0.0e	0.8±0.0cd	32±0.4e
25% of LC_{50}	5.54	52±4c	1.6±0.1c	121±0c	5.34	22±2d	0.9±0.1d	70±0.5d	24.89	29±1.5d	1.0±0.0bc	38±0.1d
33% of LC_{50}	7.32	46±1d	1.6±0.0c	128±0b	7.05	25±1c	1.1±0.1c	95±0.3c	32.86	60±0.0b	1.7±0.0ab	92±0.3c
50% of LC_{50}	11.09	74±3a	2.3±0.1a	184±0a	10.68	30±2b	1.3±0.0b	110±0.2b	49.79	69±1.1a	1.8±0.0a	132±0.6a

Means with similar letters in a column for each fish species are statistically non-significant at P<0.05.

that of *Labeo rohita*, *Ctenopharyngodon idella* and *Catla catla* (Table 3).

Cumulative Tail Length of Comets (µm): Four fish species responded differently for their sensitivity to induce DNA damage determined in-terms of cumulative tail length of comets. All the four fish species showed significantly higher sensitivity towards As exposure. *Labeo rohita* and *Cirrhina mrigala* exhibited significantly least sensitivity towards Cu exposure while both *Catla catla* and *Ctenopharyngodon idella* were less sensitive to Zn as it caused significantly low degree of DNA damage observed in-terms of cumulative tail length of comets. Mean sensitivity of four fish species, towards three metals, varied significantly that followed the order: *Cirrhina mrigala* > *Labeo rohita* > *Catla catla* > *Ctenopharyngodon idella* (Table 3).

DISCUSSION

Due to growing number of agricultural, commercial and industrial chemicals, the rate of genetic disorders, diseases and mortality of exposed organisms in the natural habitats has increased significantly (Livingstone, 2001). This needs to study the impacts of these chemicals on integrity and functioning of cellular DNA in organisms.

During present investigation all the four fish species showed concomitant increase in DNA damage in their peripheral erythrocytes with increase in metallic ion concentration. The fish, *Oreochromis mossambicus*

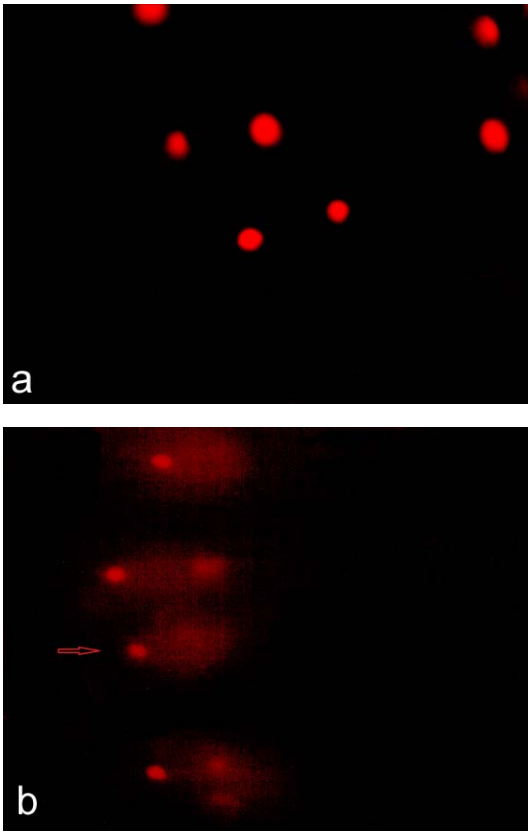

Fig. 1: Peripheral erythrocytes of control (a) and metal exposed (b) fish. Damaged cells are with Comet (arrow).

Table 3: Comparison of genotoxic damage caused by metals in peripheral erythrocytes of fish

	Exposure metals			Overall means
	As	Cu	Zn	
Percentage of damaged cells (%)				
Labeo rohita	44±2a	37±24 c	39±33b	40±3b
Cirrhina mrigala	57±31a	45±29 c	54±39 b	52±6a
Catla catla	33±25ab	32±28 b	24±17c	29±4d
Ctenopharyngodon idella	43±24a	25±17c	38±27b	35±9c
Overall Means	44±10a	35±09c	39±12b	
*Genetic Damage Index (GDI)				
Labeo rohita	1.3±0.7a	1.2±0.7b	1.3±0.9a	1.3±0.1b
Cirrhina mrigala	1.8±10a	1.5±0.9bc	1.6±1b	1.6±0.3a
Catla catla	1.2±0.8a	1.2±0.8a	0.9±.90.5b	1.1±0.1c
Ctenopharyngodon idella	1.4±0.7a	0.9±0.5c	1.2±0.6b	1.2±0.2bc
Overall Means	1.4±0.3a	1.2±0.2b	1.2±0.3b	
Cumulative Tail Length (µm)				
Labeo rohita	112±67a	104±75b	97±80c	104±7b
Cirrhina mrigala	193±128a	127±79c	136±104b	152±35a
Catla catla	101±66a	90±67b	76±43c	89±12c
Ctenopharyngodon idella	107±59a	71±44b	69±52c	82±21d
Overall Means	128±44a	98±23b	95±30c	

*GDI (Genetic Damage Index) = {Type I + 2(Type II) + 3(Type III) + 4(Type IV) / Type 0 + Type I + Type II + Type III + Type IV}; Means with similar letters in a single row are statistically non-significant at P<0.05.

exposed to different concentrations of arsenic exhibited concentration dependent increase in DNA damage in the blood cells that was significantly higher than control fish (Ahmed *et al*., 2011).

During present investigation, all the four fish species exhibited significantly higher tendency to induce DNA damage in the cells due to As exposures. However, the response of four fish species to induce GDI in their erythrocytes was significantly maximum due to exposure of As while it was minimum due to Cu exposure. Farombi *et al*. (2007) also reported As, Cu, Zn, Cd and Pb as genotoxic metals for *Clarias gariepinus*. All these metals induce oxidative stress in the fish through alteration in the antioxidant enzyme systems i.e. glutathione and induction of lipid peroxidation. Increased Al exposure has also been reported to cause time dependent increase in superoxide dismutase activity in *Ctenopharyngodon idella*. This enzyme counteracts the effect of reactive oxygen species (Van der Oost *et al*., 2003). Findings of present experiments are also in conformity with Scalon *et al*. (2010) who reported higher DNA damage in peripheral erythrocytes of a native fish species, *Hyphssobrycon luetkenii* due to Al, Cu, Cr, Ni, Zn, Fe and Pb toxicity. Kumar *et al*. (2013) also reported the genotoxic potential of As at different exposure concentrations in *Channa punctatus* and *Carassius auratus*.

Being a vertebrate model, fish is the best accessible to estimate potential risks, due to their ability to metabolize and accumulate contaminants in their bodies (Diekmann *et al*., 2004). Moreover, fish blood erythrocytes are the most suitable for DNA damage analyses since peripheral blood reflects the comprehensive health status of the organism. Regarding this issue, fish blood cells have attained particular attention as their erythrocytes are nucleated and, therefore, appropriate for obtaining nucleoids for single cell gel electrophoresis (Costa *et al*., 2011). During present investigation *Cirrhina mrigala* appeared significantly more sensitive to exhibit significantly higher mean genetic damage index, followed by that of *Labeo rohita*, *Ctenopharyngodon idella* and *Catla catla*. The abilities of various metals to cause DNA damage in terms of cumulative tail length of comets followed the order: As>Cu>Zn. Kopjar *et al.* (2008) reported significant increase in cumulative tail length of comets in peripheral erythrocytes of *Cobitis elongate* due to toxicity of industrial effluents containing As, Cu, Hg, Cr and Mn. During present experiment significantly higher percentages of tail DNA in *Cirrhina mrigala* indicate its higher susceptibility to metals, under study, than the other three fish species. All the four fish species showed variable responses towards individual metal and metal mixture toxicity due to their physiological differences and species-specificity to interact against various metals. Moreover, DNA damage caused by metals suggested a serious concern towards their potential danger to the survival of carps in the natural aquatic habitats.

Conclusion: Results showed significantly variable genotoxic damage in peripheral blood erythrocytes of all the four fish species. It is also concluded that by using Comet assay, *Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella* can suitably be used as bio-indicators of metallic ion pollution in the natural aquatic habitat. This work will also help sustainable conservation of fresh water fisheries in Pakistan.

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