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Temperature induces lead toxicity in *Pangasius hypophthalmus*: an acute test, antioxidative status and cellular metabolic stress

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Abstract Climate change and pollution are the most vulnerable stressors that are anticipated increasingly to affect all living organisms including fishes. The aquatic ecosystems are the most affected ecosystem due to contamination and global increasing temperature. In view of the above, the present study delineates 96-h median lethal concentration of heavy metal, lead alone and in combination with high temperature (34 °C) by conducting static non-renewable acute toxicity bioassay in *Pangasius hypophthalmus* (average weight 3.65 ± 0.75 g). Further, the effect of different definitive doses (80, 82, 84, 86, 88 and 90 mg/L) of lead alone and high temperature on cellular metabolic response was probed. The LC_{50} of lead was found to be 84.93 mg/L, whereas in combination with high temperature it was 83.10 mg/L in *P. hypophthalmus*. Catalase, superoxide dismutase and glutathione-S-transferase were noticeably higher ($p < 0.01$) in liver, gill and brain during lead exposure alone and in combination with high temperature. The activities of aspartate aminotransferase and alanine aminotransferase were significantly enhanced ($p < 0.01$) in muscle, liver and gill in dose- and time-dependent manners in lead-alone-exposed and in combination with high-temperature groups. The brain and liver acetylcholine esterase activities showed noticeable ($p < 0.01$) inhibition from 80 to 90 mg/L exposure of lead alone and with concurrent exposure to temperature than the control group. Overall results clearly indicate that acute exposure of lead and high temperature led to pronounced deleterious alterations on cellular and metabolic activities of *P. hypophthalmus*.

Keywords Acute toxicity · Acetylcholine esterase · Carbohydrate metabolic enzymes · Protein metabolic enzymes · *Pangasius hypophthalmus* · Oxidative stress

Introduction

Climate change due to shifting of temperature patterns and contamination of heavy metals are of great concern in light of aquatic water pollution. The toxicity of heavy metal depends on the organisms tolerable limit and beyond which it becomes toxic. In the aquatic water bodies, rising temperature enhances the toxicity of chemical contaminant (Patra et al. 2007) to the fishes. The major route of entering heavy metals in aquatic ecosystem is mainly through atmospheric interference such as deposition, erosion of geological matrix and anthropogenic activities originated by discharge of industrial effluents, domestic sewage, as well as disposal of mining wastes (Hariharan et al. 2016). Due to non-biodegradable nature of heavy metal, it persists for long time in aquatic ecosystems, and hence, it becomes major source of pollution creating constituent to natural ecosystems resulting in risk to human being and aquatic organisms including fish (Miretzky et al. 2004; Nair et al. 2006). The contaminations through heavy metal in aquatic ecosystems mainly present in soluble or suspension form and eventually tends to settle down to bottom or it may bioaccumulate in aquatic organisms especially fishes (Kumar et al. 2017a, b). Further, bioaccumulation in the fish tissue ultimately affects the

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health status of human beings after its consumption (Laxmi Priya et al. 2011). The accumulation of heavy metals in fish, pond or river water enhanced by upsurge in temperature; therefore, it is absolutely imperative to study the detrimental effects of heavy metals alone and in combination with temperature, so as to formulate the management action plans for safeguarding aquatic organisms. Moreover, pertinent information related to effects of concomitant exposure of heavy metal and elevated temperature on aquatic organism especially in fishes is still scarce.

Lead (Pb) belongs to a class of major toxic heavy metals that lead to impart contamination of aquatic ecosystem owning to its occurrence in both physical and chemical forms, which adversely affects the health status of fish at concentration higher than normal. The major source of Pb contamination is through mining, smelting, industrial effluents and anthropogenic activities through which emissions are released which play a major role in global distribution (Sorensen 1991; Heath 1995; Hariharan et al. 2016). Lead (Pb) exists mainly in inorganic form and also in different oxidation states; hence, it leads to induce oxidative stress in aquatic organism especially in fishes and bioaccumulate in different parts of fish tissues (Jackson et al. 2005; AFS 2008). Although Pb is very toxic to fishes at higher level, but lower level also creates muscular and neurological degeneration and destruction, growth inhibition, mortality, as well as reproductive problems (Rabitto et al. 2005). The levels of Pb toxicity in fishes depend upon many factors including age, pH and hardness of the water (Nussey et al. 2000).

Temperature plays vital role in regulating key physio-biochemical process (Kumar et al. 2012, 2014a, b, c, 2016a, b, c, d), and beyond certain limit of its rise, it may influence the toxicity of heavy metals through their degradation and volatilization rates, in addition to affecting the absorption and desorption processes and detoxification rates in exposed organisms (Howe et al. 1994). Rising temperature is sole culprit for increased metabolic rate that leads to increase bioaccumulation of heavy metal in organisms (Kumar et al. 2017a; Martin and Caughtrey 1976) and bioconcentration in food chain, thus resulting into deleterious affects on whole ecosystem largely due to receiving acid precipitation as well as increased solubility and bioavailability of chemicals (Yang et al. 2006; Ling et al. 2007).

The literature pertaining to the interactive effects of high temperature and acute exposure of heavy metal on enzymes activities of fish is scanty. Furthermore, effects of temperature and contamination (inorganic and organic) exposure on fish generate reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and the hydroxyl radical

(Livingstone et al. 1990). The imbalance between production and elimination of ROS causes oxidative stress which can be detoxified by an enzyme defense system, comprising superoxide dismutase (SOD) and catalase (CAT), while organic peroxides can be detoxified by the activity of glutathione-S-transferase (Halliwell and Gutteridge 1999). The antioxidant enzymes, neurotransmitter enzymes (acetylcholine esterase), carbohydrate metabolic enzymes (LDH and MDH) and protein metabolic enzymes (ALT and AST) are used as biomarkers to monitor the stress condition in aquatic organism especially fish (Orbea et al. 2002; Kumar et al. 2014a, b, c, 2016a, b, c, d).

To the best of understanding, effects of acute toxicity of Pb alone and in combination with high temperature in terms of biochemical stress markers in *Pangasius hypophthalmus* remain to be identified; hence, the present study was aimed to determine lethal concentration (LC$_{50}$) of Pb alone and in combination with high temperature for 96 h to assess exposure of *Pangasius hypophthalmus* in terms of cellular metabolic stress.

The experiment was conducted at ICAR-National Institute of Abiotic Stress Management, Baramati, Pune, Maharashtra, India, during August 04–28, 2016, in wet laboratory, and biochemical analysis was carried out during August 30 to September 30, 2016.

Materials and methods

Experimental animals

Pangasius hypophthalmus fingerlings were procured from the local fish market (Nil Aquarium, Baramati, Pune, India) having an average weight of 3.65 ± 0.75 g. The fish were quarantine with prophylactic dip in (1%) salt solution for 5 min and then acclimatized in the fiber-reinforced plastic tanks (circular, 500 L) for period of 15 days prior to commencement of the experiment. Aeration was provided throughout the experimental period with compressed air pump. The animals were fed twice with practical diet (30% crude protein) @ of 3% of their body weight, and feeding was stopped prior to 48 h of LC$_{50}$ experiment. The water quality parameters were recorded within the normal range for culture of *P. hypophthalmus* (Kumar et al. 2011a, b, 2014a, b, c, 2016a, b, c, d).

Median lethal concentration

The static non-renewable acute toxicity bioassay for 96 h was performed to determine lethal concentration (LC$_{50}$) of Pb alone and in combination with high temperature (34 °C)

in *P. hypophthalmus* as per standard method of APHA (1998). The lead nitrate (Pb NO$_3$)$_2$ (Merck, Pune, India) was used for lethal concentration experiment, and 100 ppt (parts per thousand) of stock solution was prepared for use during experiment. The test concentration was determined with the help of range finding test, and further, definitive test was performed for final determination of LC$_{50}$ concentration of Pb. The range finding test was chosen in the following concentration, viz. 20, 40, 60, 80, 100 and 120 mg/L with four fish in each replicate, and further, range finding test was performed at concentration, viz. 80, 90 and 100 mg/L. The range of LC$_{50}$ of Pb for *P. hypophthalmus* was found between 80 and 90 mg/L. Therefore, the definitive test concentrations such as 80, 82, 84, 86, 88 and 90 mg/L were performed in triplicates with 10 fish in each replicate. Same concentrations were also used with high temperature (34 °C) to evaluate the LC$_{50}$ in concurrent exposure to temperature and Pb in *P. hypophthalmus*. The cumulative mortality (%) was also recorded during 24, 48, 72 and 96 h interval.

Sample preparation for enzymatic analysis

Lived fish were anesthetized with clove oil @ 100 mg/L, and thereafter, fish were dissected under aseptic condition to collect muscle, gill, liver and brain tissues. Tissues were homogenized (5% w/v) with chilled sucrose solution (0.25 M) in a glass tube using Teflon-coated mechanical tissue homogenizer (Omni Tissue Master Homogenize, Kennesaw, GA). The tube was kept on ice to avoid denaturation of the enzymes during the homogenization. The homogenates were centrifuged at 5000 rpm for 20 min at 4 °C in a cooling centrifuge. Protein contents in the supernatants were quantified following the method of Lowry et al. (1951).

Antioxidant enzymes

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured by the method of Misra and Fridovich (1972). The assay was based on the oxidation of epinephrine–adrenochrome transition by the enzyme. Catalase (CAT) (EC 1.11.1.6) activity was measured by the method of Takahara et al. (1960). The solution of 2.45 mL phosphate buffer (50 mM; pH-7), 50 μL tissue homogenate and 1 mL of hydrogen peroxide substrate solution (freshly prepared) are mixed well and observed reading at 240 nm for 3 min. Gluthathione-s-transferase (GST) (EC 2.5.1.18) was measured spectrophotometrically by the method of Habing

et al. (1974). The S-2, 4-dinitrophenyl glutathione (CDNB) was used as a substrate.

Acetylcholine esterase (AChE)

AChE (EC. 3.1.1.7) activity was measured using the method of Hestrin modified by Augustinsson (1949). The activity was spectrophotometrically measured as the increase in absorbance of the sample at 540 nm.

Carbohydrate metabolic enzymes

Lactate dehydrogenase (LDH; L-lactate NAD1 oxidoreductase; EC.1.1.1.27) was assayed using 0.1 M phosphate buffer (pH 7.5) and 0.2 mM NADH solution in 0.1 M phosphate buffer. The reaction was initiated with the addition of substrate 0.2 mM sodium pyruvate, and absorbance was recorded at 340 nm (Wroblewski and LaDue 1955). A similar reaction mixture like LDH was used for the estimation of malate dehydrogenase (MDH; L-malate: NAD$^+$ oxidoreductase: EC.1.1.1.37) except for the substrate (1 mg oxaloacetate/mL of chilled triple distilled water) (Ochoa 1955).

Protein metabolic enzymes

Aspartate aminotransaminase (AST; EC.2.6.1.1) and alanine aminotransaminase (ALT; EC.2.6.1.2) activities were measured by the estimation of oxaloacetate and pyruvate released, respectively, after incubating the reaction mixture at 37 °C for 60 min (Wootton 1964).

Sample preparation for metal analysis

The water samples were kept in freeze at 4 °C and after one week of LC$_{50}$ experiment proceeded for Pb analysis. The water samples were filtered with 0.45-μm pore size filter papers, and acidic pH of water samples was made with 100 μL of pure HNO$_3$ (69%, Himedia Laboratory Pvt. Ltd., Mumbai, India). For Pb concentration analysis in muscle tissue, 0.3–0.5 g of samples was collected from *P. hypophthalmus*. The muscle samples were processed for acidic digestion in microwave digestion system (Microwave Digestion System, Model START-D, SN-135177, Milestone, USA). HNO$_3$ and H$_2$O$_2$ were added in 5:1 ratio for digestion, and then completely digested samples were allowed to cool to room temperature. Later on, each digested samples were filtered with 0.45-μm pore size filter paper and volume was made up to 50 mL to proceed further for metal

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Table 1 Median lethal concentration (LC$_{50}$) and cumulative mortality (%) of *Pangasius hypophthalmus* exposed to different concentrations of lead (Pb) alone and in combination with temperature (34 °C) for period of 96 h

Period of exposure (h)	R² Value	LC$_{50}$ (mg/L)	95% confidence interval		S value	Safe level	Intercept	Slope	Cumulative mortality (%)					
			Lower	Higher					80 (mg/L)	82 (mg/L)	84 (mg/L)	86 (mg/L)	88 (mg/L)	90 (mg/L)
Experiments conducted at environmental temperature (26–28 °C)														
24	0.78	91.70	89.29	98.22	1.04	24.48	−5.07	0.09	20.00	20.00	23.33	26.67	30.00	30.00
48	0.88	88.20	86.65	91.12			−6.53	0.12	36.67	33.33	40.00	43.33	60.00	60.00
72	0.76	86.55	85.11	88.68			−12.01	0.20	43.33	40.00	43.33	53.33	73.33	73.33
96	0.87	84.93	83.48	86.40			−15.63	0.25	50.00	46.67	53.33	63.33	83.33	83.33
Treated with 34 °C temperature														
24	0.75	99.93	91.95	111.23	1.14	20.19	−0.41	0.02	13.33	16.67	13.33	23.33	13.33	20.00
48	0.85	87.59	85.78	91.35			−14.60	0.21	20.00	33.33	33.33	50.00	33.33	50.00
72	0.87	84.78	82.79	86.74			−13.26	0.21	30.00	46.67	53.33	60.00	50.00	60.00
96	0.79	83.10	80.80	84.61			−14.36	0.24	40.00	63.33	66.67	70.00	63.33	73.33

analysis through inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700 series, Agilent Technologies, USA). The multielement standard solutions of 10 µg/mL were used to prepare calibration curve. The calibration curves with $R^2 > 0.999$ were accepted for concentration calculation (Kumar et al. 2017b; 2017c).

Statistics

The data were statistically analyzed by Statistical Package for the Social Sciences (SPSS) version 16. The one-way ANOVA (analysis of variance) was used to see the treatment effect. Duncan's multiple range tests were used to see the significant difference between the mean if any, and the comparisons were made at the 5 and 1% probability level. The probit analysis was used for LC$_{50}$ determination using, Basic LD$_{50}$ version 1.1 (Trevors 1986).

Results and discussion

Lethal concentration (LC$_{50}$) of heavy metal Pb alone and in combination with high temperature (34 °C) and cumulative mortality (%) at respective concentration of 24, 48, 72 and 96 h LC$_{50}$ at 95% confidence limits in *P. hypophthalmus* are presented in Table 1. The LC$_{50}$ values of Pb alone at 24, 48, 72 and 96 h were found to be 98.22, 91.12, 88.68 and 86.40 mg/L, respectively, whereas LC$_{50}$ of Pb in combination with high temperature (34 °C) was found to be 111.23, 91.35, 86.74 and 84.61 mg/L during 24, 48, 72 and 96 h, respectively. The cumulative mortalities were observed in range of 20–50, 20–46, 23–53, 26–63, 30–83 and 30–83% at 80, 82, 84, 86, 88 and 90 mg/L concentration, and in combination with Pb- and high-temperature-exposed groups it was found to be 13–40, 16–63, 13–66, 23–70 and 20–73% during 24, 48, 72 and 96 h, respectively. We obserbed several behavioral changes in the *P. hypophthalmus* during acute toxicity exposure, such as hyperactivities, altered movements, trying to escape from tanks. Some of the fishes change their body position to vertical for few minute with the anterior side up and trying to gulp the atmospheric oxygen and tail pointed in downward direction, finally settled in the bottom of the tank in upside down position and opercular movement was ceased and eventually fish was declared died.

The results of the present study indicated that acute toxicity (LC$_{50}$ at 96 h) of Pb increased at higher temperature (34 °C) as it was found to be 84.61 mg/L, while the acute toxicity of Pb alone was 86.40 mg/L; hence, it is proved that elevated temperature induces more toxicity to heavy metal exposure. The combined effect of Pb and high temperature might have increased diffusion or active uptake of water and solute across the gills or other cell

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membrane of the fish. Gill is considered as one of the main target organs to perform filtering of water including all kinds of toxicants. Temperature coefficients for free diffusion of solute molecules through gill are 1.3–1.4 but for permeability through cellular membranes may be considerably larger than this size, and because of this reason, temperature might have induced increased toxicity (Giese 1963). Apart from this, toxicity is also influenced by several others factors such as aquatic environment condition, chemical and physical parameters. The solubility of dissolved oxygen concentration decreases with consequent increase in temperature, and aquatic organisms might survive at this condition, but further addition of contamination becomes unbearable to them (Cairns et al. 1975). This fact is attested by the results of the present study wherein safe concentration ranged from 1.04 to 24.24 mg/L in Pb alone exposure, while in concurrent exposure to Pb and high temperature it was 1.14–20.19 mg/L.

Stress biomarker enzymes

Antioxidative enzymes

The antioxidative status (catalase, SOD and GST) in liver, gill and brain of *P. hypophthalmus* exposed to Pb alone and concurrent exposure to Pb and high temperature (34 °C) for 96 h is presented in Table 2. The catalase, SOD and GST were noticeable ($p < 0.01$) higher during exposure to both stressors in dose- and time-dependent manner in comparison with control group. The catalase activities in liver, gill and brain showed remarkably higher activities in Pb-exposed group compared to control, and also the catalase activities were significantly higher ($p < 0.01$) in concurrent exposure to Pb and high temperature compared to control and Pb-alone-exposed group. The catalase activities in liver, gill and brain showed enhancement of 36–125, 9–70 and 21–67%, respectively, in the group exposed to 80–90 mg/L Pb alone; similarly, catalase activities varied from 81 to 109, 52 to 102 and 34 to 41% in liver, gill and brain, respectively, on exposure to Pb and high temperature in comparison with Pb-alone-exposed group. In case of SOD, the activities in liver, gill and brain were significantly higher ($p < 0.01$) in comparison with control group. The SOD activities ranged from 61 to 73 and 20 to 28 in liver and gill, respectively, on exposure to 80–90 mg/L of Pb; similarly, the SOD activities in liver and gill ranged from 6 to 8 and 0.8 to 44% on concurrent exposure to Pb and temperature group in comparison with

Pb-alone-exposed group. The GST activities in liver, gill and brain were also remarkably ($p < 0.01$) higher in Pb-exposed group as well as in the concurrent exposure to Pb and temperature group. The GST activities ranged from 59 to 109, 86 to 175 and 49 to 63 in liver, gill and brain, respectively, on exposure group of 80–90 mg/L of Pb; similarly, on simultaneous exposure to Pb and high-temperature group, activities ranged from 30 to 46, 46 to 86 and 11 to 34% in liver, gill and brain, respectively, as compared to Pb-exposed group.

The oxidative stress enhanced after exposure to single stressor and the intensity of stress further enhanced by multiple stressors. This might be attributable due to the generation of hydrogen peroxide at mitochondrial levels by superoxide dismutase and their elimination by catalase, glutathione and peroxiredoxin–thioredoxin systems (Di Giulio and Meyer 2008; Lushchak 2011). Due to the production of oxygen free radicals (OFRs), the cellular and functional protein becomes potential target of oxidative damage (Bainy et al. 1996). Therefore, to neutralize activities of OFRs, combined action of both enzymatic and non-enzymatic antioxidant is valuable (Lopez-Torres et al. 1993). To support these findings, previous studies reported enhanced oxidative stress in *Labeo rohita*, *Oreochromis mossambicus* and *Chanos chanos* exposed to organic contaminant (Kumar et al. 2011a, 2011b, 2014a, b, c, 2016a, b, c, d) and elevated oxidative stress post-temperature exposure to pre-acclimated fish with organic contamination have been reported (Kumar et al. 2014a, b, c, 2016a, b, c, d). The oxidative stress gets intensified after exposure to multiple stresses in fish because the balance between oxidant (free radical and non-radical) and antioxidant machinery is disrupted due to alteration in biomolecules such as protein, lipid, carbohydrate and nucleic acid. The reactive oxygen species mainly hydrogen peroxide can alter reactive amino acid cysteine residues within proteins, converting them from S–H (thiol) to S–OH (sulphenic) derivatives (Finkel 2003).

Enzymes of protein metabolism

The protein metabolic enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of *P. hypophthalmus* exposed to Pb alone and in combination with temperature are presented in Table 3. AST activities in muscle, liver and gill were significantly higher ($p < 0.01$) in Pb-exposed group as well as in Pb and temperature-exposed group. With the gradual increase in dose of Pb alone and in combination with high temperature

Table 2 Catalase, SOD and GST activities in *Pangasius hypophthalmus* exposed to different concentrations of lead (Pb) alone and in combination with temperature (34 °C) for period of 96 h

Treatments	Catalase			Superoxide dismutase (SOD)			Glutathione-S-transferase (GST)		
	Liver	Gill	Brain	Liver	Gill	Brain	Liver	Gill	Brain
0 (Control)	$19.00^a \pm 0.90$	$19.68^a \pm 0.74$	$20.75^a \pm 0.53$	$36.98^a \pm 2.04$	$28.14^a \pm 0.56$	$55.82^a \pm 0.50$	$1.00^a \pm 0.06$	$1.05^a \pm 0.04$	$0.89^a \pm 0.04$
Pb-80.0	$25.85^b \pm 0.94$	$21.50^{ab} \pm 2.18$	$25.14^b \pm 0.84$	$59.69^b \pm 1.18$	$33.85^b \pm 1.12$	$71.47^d \pm 3.19$	$1.60^b \pm 0.03$	$1.96^b \pm 0.16$	$1.32^c \pm 0.06$
Pb-82.0	$27.06^b \pm 0.14$	$23.12^{ab} \pm 1.23$	$25.96^b \pm 0.73$	$58.80^b \pm 1.14$	$36.18^c \pm 2.53$	$65.66^b \pm 0.32$	$1.75^b \pm 0.02$	$2.22^{bc} \pm 0.17$	$1.37^c \pm 0.03$
Pb-84.0	$27.34^b \pm 0.84$	$21.29^{ab} \pm 0.54$	$27.43^{bc} \pm 0.67$	$62.21^c \pm 0.76$	$33.74^b \pm 1.08$	$64.11^b \pm 0.49$	$1.71^b \pm 0.01$	$2.67^d \pm 0.10$	$1.16^b \pm 0.03$
Pb-86.0	$35.41^c \pm 0.85$	$22.59^{ab} \pm 0.64$	$26.51^b \pm 1.07$	$59.66^b \pm 0.80$	$34.46^b \pm 2.26$	$70.01^d \pm 0.49$	$1.71^b \pm 0.03$	$2.47^c \pm 0.17$	$1.35^c \pm 0.02$
Pb-88.0	$35.61^c \pm 0.83$	$24.22^b \pm 1.45$	$30.66^c \pm 3.04$	$59.47^b \pm 1.46$	$39.18^d \pm 0.34$	$67.10^c \pm 0.73$	$2.04^c \pm 0.09$	$2.31^{bc} \pm 0.07$	$1.31^c \pm 0.05$
Pb-90.0	$42.86^d \pm 0.47$	$33.54^c \pm 0.78$	$34.85^d \pm 0.74$	$64.07^c \pm 0.71$	$36.25^c \pm 1.49$	$70.56^d \pm 0.58$	$2.10^c \pm 0.03$	$2.89^e \pm 0.06$	$1.45^d \pm 0.07$
Pb + T-80.0	$46.98^e \pm 1.20$	$32.73^c \pm 0.95$	$33.82^d \pm 0.61$	$63.42^c \pm 0.68$	$34.15^b \pm 3.16$	$70.35^d \pm 1.50$	$2.10^c \pm 0.05$	$2.88^e \pm 0.24$	$1.48^d \pm 0.02$
Pb + T-82.0	$46.52^e \pm 0.70$	$31.84^c \pm 0.40$	$34.98^d \pm 1.02$	$62.63^c \pm 0.63$	$35.26^{bc} \pm 2.00$	$69.28^{cd} \pm 1.07$	$2.09^c \pm 0.03$	$3.12^f \pm 0.18$	$1.53^e \pm 0.03$
Pb + T-84.0	$46.63^e \pm 0.22$	$40.29^e \pm 1.72$	$33.49^d \pm 0.57$	$63.84^c \pm 1.70$	$32.26^b \pm 1.01$	$73.59^e \pm 0.76$	$2.05^c \pm 0.12$	$3.64^h \pm 0.17$	$1.43^d \pm 0.04$
Pb + T-86.0	$53.20^g \pm 0.50$	$37.59^d \pm 0.77$	$35.17^e \pm 0.40$	$59.72^b \pm 0.49$	$38.48^{cd} \pm 2.05$	$67.67^c \pm 0.74$	$2.34^d \pm 0.04$	$2.83^e \pm 0.13$	$1.90^f \pm 0.03$
Pb + T-88.0	$50.41^f \pm 1.09$	$35.31^d \pm 1.19$	$32.46^d \pm 1.13$	$63.63^c \pm 2.20$	$40.37^e \pm 1.20$	$70.50^d \pm 0.65$	$2.29^d \pm 0.07$	$3.22^g \pm 0.09$	$1.83^f \pm 0.03$
Pb + T-90.0	$54.17^g \pm 1.44$	$43.53^f \pm 1.46$	$35.65^e \pm 1.69$	$64.76^d \pm 0.67$	$48.74^e \pm 1.40$	$78.17^f \pm 0.24$	$2.35^d \pm 0.03$	$3.65^h \pm 0.21$	$1.78^{ef} \pm 0.04$
p Value	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

Lead (Pb) (mg/L)/temperature (T) (34 °C), values in the same column with different superscripts differ significantly ($p < 0.01$). Data expressed as mean $\pm$ SE ($n = 6$)

Catalase, *SOD* superoxide dismutase, *GST* glutathione-S-transferase, Units/mg protein

Table 3 Aspartate aminotransferase and alanine aminotransferase activities in *Pangasius hypophthalmus* exposed to different concentrations of lead (Pb) alone and in combined with temperature (34 °C) for a period of 96 h

Treatments	Aspartate aminotransferase (AST)			Alanine aminotransferase (ALT)		
	Muscle	Liver	Gill	Muscle	Liver	Gill
0 (Control)	3.95[a] ± 0.44	6.34[a] ± 0.97	5.91[a] ± 0.67	6.20[a] ± 0.83	10.16[a] ± 0.56	8.75[a] ± 1.14
Pb-80.0	5.84[b] ± 0.33	9.46[b] ± 0.39	13.28[b] ± 0.58	9.96[b] ± 1.24	13.99[ab] ± 0.53	15.11[b] ± 0.84
Pb-82.0	6.41[b] ± 0.76	13.55[c] ± 1.03	13.91[b] ± 1.07	11.97[bc] ± 0.65	13.80[ab] ± 0.25	17.76[b] ± 0.38
Pb-84.0	8.52[c] ± 0.18	14.72[c] ± 1.13	14.80[b] ± 2.05	13.58[c] ± 0.76	17.97[b] ± 0.30	18.89[bc] ± 1.33
Pb-86.0	8.31[c] ± 0.96	17.71[d] ± 0.97	17.13[bc] ± 1.14	13.36[c] ± 0.95	18.40[b] ± 0.25	18.29[bc] ± 1.24
Pb-88.0	12.21[d] ± 1.28	18.07[d] ± 1.78	20.57[c] ± 1.77	18.33[d] ± 0.21	19.43[b] ± 1.40	19.77[c] ± 0.43
Pb-90.0	15.55[e] ± 0.80	22.23[e] ± 2.0	27.03[d] ± 2.36	19.49[d] ± 0.80	19.39[b] ± 0.33	21.55[cd] ± 1.15
Pb + T-80.0	20.34[f] ± 0.84	28.63[f] ± 0.73	29.82[de] ± 0.77	26.85[f] ± 1.59	23.82[c] ± 1.22	23.53[d] ± 1.25
Pb + T-82.0	14.43[de] ± 0.21	24.33[ef] ± 0.29	32.67[e] ± 1.62	22.28[e] ± 0.95	22.86[c] ± 1.01	23.86[d] ± 1.18
Pb + T-84.0	17.64[ef] ± 2.07	25.95[ef] ± 0.82	33.94[e] ± 1.49	24.95[ef] ± 1.39	24.87[c] ± 1.57	27.66[e] ± 0.41
Pb + T-86.0	20.27[f] ± 0.92	27.99[ef] ± 1.05	29.98[de] ± 0.82	29.59[fg] ± 0.42	26.34[d] ± 0.48	25.00[de] ± 1.59
Pb + T-88.0	21.92[f] ± 1.75	25.62[ef] ± 2.86	31.20[e] ± 0.45	29.68[fg] ± 0.88	25.65[cd] ± 0.74	29.28[f] ± 1.23
Pb + T-90.0	26.23[g] ± 0.72	28.72[f] ± 0.73	37.86[f] ± 1.11	33.94[g] ± 1.25	28.59[e] ± 0.61	33.70[g] ± 0.55
p Value	*p* < 0.01	*p* < 0.01	*p* < 0.01	*p* < 0.01	*p* < 0.01	*p* < 0.01

Lead (Pb) (mg/L)/temperature (T) (34 °C), values in the same column with different superscript differ significantly ($p < 0.01$). Data expressed as mean ± SE ($n = 6$). Aspartate aminotransferase: nmole of sodium pyruvate formed/mgprotein/min at 37 °C, AST: nmole oxaloacetate released/min/mgprotein at 37 °C

Table 4 Lactate dehydrogenase and malate dehydrogenase activities in *Pangasius hypophthalmus* exposed to different concentrations of lead (Pb) alone and in combination with temperature (34 °C) for period of 96 h

Treatments	Lactate dehydrogenase (LDH)			Malate dehydrogenase (MDH)		
	Muscle	Liver	Gill	Muscle	Liver	Gill
0 (Control)	0.84[a] ± 0.06	0.28[a] ± 0.03	0.73[a] ± 0.06	0.26[a] ± 0.01	0.28[a] ± 0.02	0.21[a] ± 0.02
Pb-80.0	1.66[b] ± 0.14	0.51[b] ± 0.04	0.91[ab] ± 0.02	0.45[b] ± 0.02	0.39[ab] ± 0.01	0.29[ab] ± 0.01
Pb-82.0	2.24[c] ± 0.52	0.72[c] ± 0.04	1.17[b] ± 0.14	0.49[b] ± 0.03	0.44[b] ± 0.01	0.31[ab] ± 0.02
Pb-84.0	2.17[c] ± 0.04	0.79[c] ± 0.03	1.29[b] ± 0.15	0.43[b] ± 0.01	0.44[b] ± 0.01	0.38[b] ± 0.03
Pb-86.0	3.27[e] ± 0.27	0.93[cd] ± 0.02	1.31[b] ± 0.07	0.47[b] ± 0.04	0.48[b] ± 0.03	0.46[bc] ± 0.02
Pb-88.0	2.79[d] ± 0.25	1.07[d] ± 0.11	1.43[bc] ± 0.27	0.64[bc] ± 0.03	0.50[b] ± 0.02	0.48[bc] ± 0.03
Pb-90.0	3.47[e] ± 0.15	1.24[de] ± 0.15	1.56[c] ± 0.12	0.58[bc] ± 0.03	0.53[bc] ± 0.03	0.53[c] ± 0.03
Pb + T-80.0	4.26[f] ± 0.61	1.66[e] ± 0.06	1.86[d] ± 0.13	0.64[bc] ± 0.05	0.62[bc] ± 0.04	0.64[d] ± 0.02
Pb + T-82.0	4.37[f] ± 0.08	1.91[f] ± 0.43	1.39[b] ± 0.08	0.66[c] ± 0.02	0.56[bc] ± 0.05	0.71[e] ± 0.04
Pb + T-84.0	4.41[f] ± 0.21	1.76[e] ± 0.49	1.35[b] ± 0.16	0.71[c] ± 0.08	0.62[bc] ± 0.01	0.65[d] ± 0.02
Pb + T-86.0	3.57[ef] ± 0.13	2.36[g] ± 0.26	1.43[bc] ± 0.13	0.63[bc] ± 0.16	0.64[bc] ± 0.03	0.65[d] ± 0.04
Pb + T-88.0	4.35[f] ± 0.19	1.82[f] ± 0.73	1.86[d] ± 0.17	0.87[d] ± 0.02	0.62[bc] ± 0.03	0.67[d] ± 0.03
Pb + T-90.0	5.29[g] ± 0.29	3.03[h] ± 0.21	2.73[e] ± 0.26	1.09[e] ± 0.12	0.67[c] ± 0.02	0.69[e] ± 0.05
p Value	*p* < 0.01	*p* < 0.01	*p* < 0.01	*p* < 0.01	*p* < 0.01	*p* < 0.01

Lead (Pb) (mg/L)/temperature (T) (34 °C), values in the same column with different superscript differ significantly ($p < 0.01$). Data expressed as mean ± SE ($n = 6$). Lactate dehydrogenase and malate dehydrogenase units/min/mg protein at 37 °C

(34 °C), the activity of AST varied from 47 to 293, 124 to 357 and 49 to 250% in muscle, liver and gill, respectively, on exposure to 80–90 mg/L of Pb in comparison with control. Similarly, with concurrent exposure to Pb and temperature, the AST activity ranged from 248 to 564, 124 to 540 and 202 to 353% in muscle, liver and gill, respectively, on exposure to Pb and high temperature in comparison with Pb exposure group. The ALT activities in muscle, liver and gill ranged from 60 to 214, 72 to 146 and 37 to 90%, respectively, on exposure to 80–90 mg/L of Pb

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Fig. 1 a, b Acetyl choline esterase activities of *Pangasius hypophthalmus* exposed to different concentrations of lead (Pb) alone and in combination with temperature (34 °C) for period of 96 h. *Values* with *different superscripts (a, b, c, d, e)* on the *top* of *bar* differ significantly ($p < 0.01$). Data expressed as mean ± SE ($n = 6$)
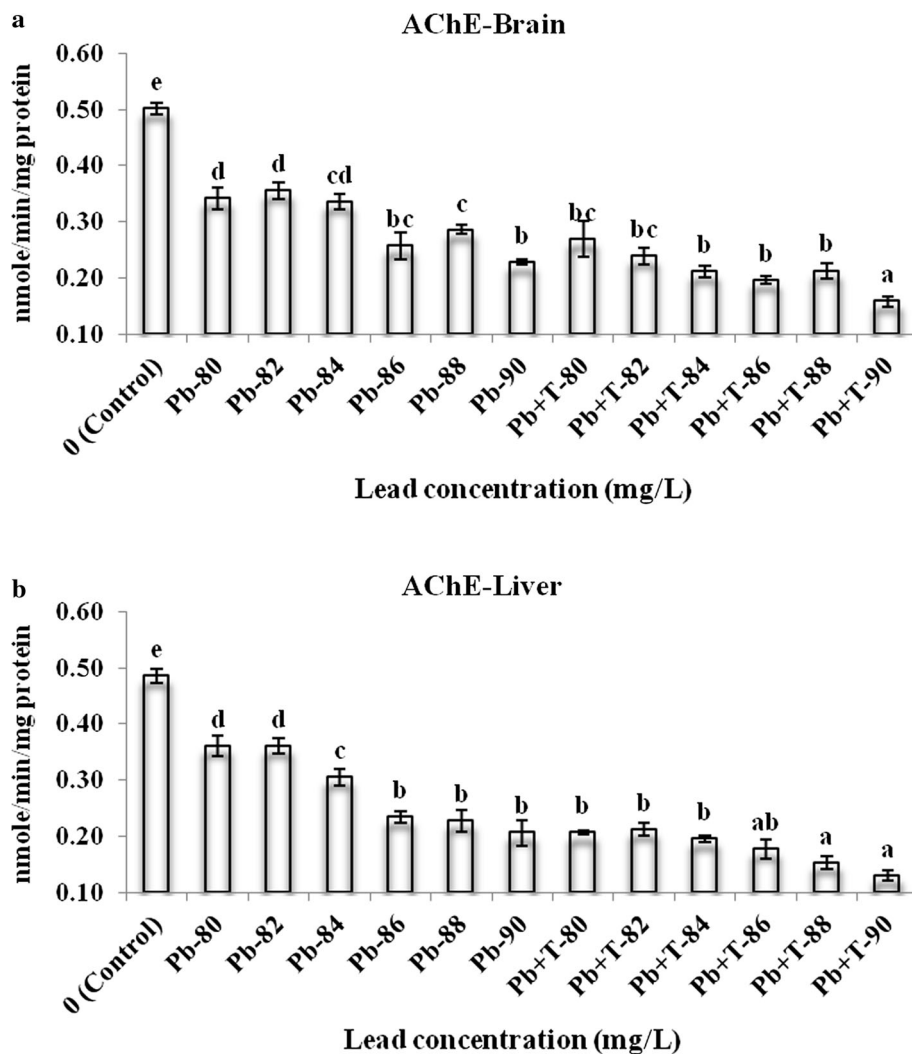
in comparison with control group. Further, the ALT activities in simultaneous exposed Pb and temperature group ranged from 169 to 447, 55 to 285 and 70 to 181% in muscle, liver and gill, respectively, in comparison with Pb-exposed group.

AST and ALT are prominent stress biomarker enzymes, used for investigating health status and more importantly for tissue damage caused by pollutants in fish (Kumar et al. 2014c). The enhanced activities of AST and ALT indicate that both enzymes are involved in gluconeogenesis process for glucose production to provide energy supply to the fish to combat stress (Kumar et al. 2014c). Results of previous studies in this laboratory also found similar observation of enhanced AST and AST activity after exposure to pollutant in fishes (Kumar et al.

2011b, Kumar et al. 2012, 2014c, 2016c, d). All kinds of pollutants are mainly stored in the liver and muscle tissue; hence, these enzymes are much affected by liver and muscle tissue condition. The elevated level of AST and ALT might be either due to hepatocellular damage or cellular degradation caused by Pb contamination (Yamawaki et al. 1986) or due to deamination of amino acid to produce tricarboxylic acid (TCA) cycle intermediates resulting in the induction of elevation in transamination pathway (Gupta et al. 2014b).

Enzymes of carbohydrate metabolism

The carbohydrate metabolic enzymes such as lactate dehydrogenase (LDH) and malate dehydrogenase (MDH)

in muscle, liver and gill of *P. hypophthalmus* exposed to Pb alone and in combination with temperature are presented in Table 4. The LDH and MDH activities in muscle, liver and gill were noticeably higher ($p < 0.01$) on exposure to Pb alone and in combination with high temperature. LDH activities were significantly enhanced ($p < 0.01$) in Pb-exposed group in time- and dose-dependent manner. The activities ranged from 98 to 313, 82 to 339 and 23 to 113% in muscle, liver and gill, respectively, on exposure to 80–90 mg/L in comparison with control group. Similarly, the LDH activities ranged from 156 to 530, 222 to 977 and 104 to 272% in muscle, liver and gill, respectively, as compared to Pb-exposed group. After exposure to Pb and temperature, the MDH activities were enormously enhanced. In case of Pb-exposed group, MDH activities in liver, gill and muscle ranged from 73 to 124, 40 to 91 and 35 to 152%, respectively, as compared to control, whereas after concurrent exposure to Pb and high temperature, the MDH activities ranged from 43 to 321, 56 to 140 and 122 to 230% in muscle, liver and gill, respectively, as compared to Pb-exposed group.

With exposure to Pb alone and in combination with high temperature, LDH and MDH were significantly elevated due to tissue damage such as liver and other vital organs. The enhanced activities showed that fish exposed to Pb alone as well as combination of Pb and high temperature induced anaerobic metabolism to maintain energy demand (Diamantino et al. 2001). To cater the energy demand, catabolic reaction might have been prompted to produce lactate resulting into elevation of both LDH and MDH (Simon et al. 1983). The major role of LDH is to convert muscle lactic acid into pyruvic acid, an essential step for producing cellular energy (Kumar et al. 2014c; 2016c). It is tempting to speculate that supply of energy requirements through anaerobic oxidation in animal under stress might have fulfilled, and therefore, the activities were enhanced.

Enzymes of neurotransmitter

The activities of neurotransmitter (acetylcholine esterase, AChE) in brain and liver of *P. hypophthalmus* are presented in Fig. 1a, b. The brain and liver AChE activities were remarkably inhibited ($p < 0.01$) in Pb-exposed group compared to control group. The inhibition of AChE activities by 31, 29, 31, 48, 42, 54% and 25, 25, 37, 51, 53, 57% was observed in the brain and liver tissue, respectively, in 80–90 mg/L of Pb-exposed group in comparison with control group. Further, on concomitant exposure to Pb and high

Table 5 Lead concentration in experimental water and muscle sample of fish

Treatments	Lead concentration in experimental water (µg/L)	Lead concentration in fish muscle (mg/kg)
0 (Control)	2.11 ± 0.37	0.02 ± 0.001
Pb-80.0	183.91 ± 1.88	10.20 ± 1.96
Pb-82.0	303.62 ± 0.59	9.47 ± 2.30
Pb-84.0	231.50 ± 0.88	6.96 ± 1.22
Pb-86.0	226.25 ± 1.13	9.61 ± 2.31
Pb-88.0	153.54 ± 1.47	9.82 ± 2.85
Pb-90.0	391.23 ± 0.67	13.13 ± 3.31
Pb + T-80.0	341.73 ± 0.74	9.85 ± 2.04
Pb + T-82.0	487.52 ± 2.23	9.71 ± 3.41
Pb + T-84.0	458.21 ± 0.39	10.33 ± 4.25
Pb + T-86.0	491.66 ± 1.58	10.87 ± 4.05
Pb + T-88.0	499.89 ± 1.22	12.08 ± 1.64
Pb + T-90.0	518.87 ± 1.77	14.32 ± 2.29

Lead (Pb) (mg/L)/temperature (T) (34 °C)

temperature the AChE activities declined by 20, 52, 57, 60, 57 68% and 42, 56, 59, 63, 68, 73% in the brain and liver tissues, respectively, in comparison with Pb exposure group.

Acetylcholine esterase (AChE) is one of the important indices in toxicity studies, hence known as biomarker enzymes for examining stress in aquatic organisms including fish (Gupta et al. 2014a). AChE catalyzes hydrolysis of acetylcholine at synaptic junctions to facilitate the nerve impulse transmission from one cholinergic neuron to another cholinergic neuron (Yadav et al. 2009) resulting into dysfunction of ion channels of neurons, particularly sodium ions after exposure to pollutants (Sogorb and Vilanova 2002). The drastic reduction in the activities of AChE might also play a vital role in behavioral changes in fish after exposure to Pb alone and in combination with temperature. The reduced AChE activities might be correlated due to changes in other enzymes function in liver (Tayebati et al. 2009; El-Demerdash 2011). Results of earlier studies also supported the present findings, wherein drastical reduction in AChE activities to contaminant exposure in fishes (Kumar et al. 2011a, 2012, 2014b, 2016b, c; Muthappa et al. 2014).

Bioaccumulation of Pb in muscle and water sample

The concentration of Pb in water sample and muscle tissue is presented in Table 5. Pb concentration in water of control group (unexposed to Pb) was 2.11 µg/L, while in Pb-exposed groups of 80, 82, 84, 86, 88 and 90 mg/L, concentration of Pb

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183, 303, 231, 226, 153, 391 µg/L, respectively, was found in water sample. The concentration of Pb was increased on concurrent exposure to Pb and temperature group, and the concentration varied from 341, 487, 458, 491, 499 and 518 µg/L in Pb-exposed group of 80, 82, 84, 86, 88 and 90 mg/L, respectively. Similarly, in the muscle tissue, the lead concentration of 0.02, 10.20, 9.47, 6.96, 9.61, 9.82 and 13.13 mg/kg was found in Pb-exposed groups of 80, 82, 84, 86, 88 and 90 mg/L, respectively. The concentration of Pb on concurrent exposure to Pb and high-temperature group was higher and found to vary in the range from 9.85 to 14.32 mg/kg in Pb-exposed group of 80–90 mg/L.

The lead concentration was found in both water and muscle tissue. The concentration of Pb was low as compared to exposure dose. The added level of Pb was in 80, 82, 84, 86, 88 and 90 mg/L, but the maximum concentration up to 518 µg/L was observed. Pb has properties to make complexes with mucus and finally convert into complex of the lead which is impermeable, and hence, it gets reduced (Luszczek-Trojnar et al. 2013), and also due to the physiological differences in the function and structure of different tissues, it may affect the bioaccumulation of metals (Kotze 1997).

Conclusion

To summarize the above findings, it is concluded that acute toxicity of heavy metal Pb is enhanced by the exposure of high temperature in *P. hypophthalmus*. Concurrent exposure of lead and high temperature led to pronounced deleterious alterations on cellular and metabolic activities of *P. hypophthalmus*. The knowledge database generated on the biomarker enzymes with exposure to Pb alone and in combination with high temperature could be utilized for aquatic ecotoxicological studies in order to formulate the suitable remedial management action plan.

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