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RESEARCH ARTICLE

TOXICITY ANALYSES OF RAW AND PHYTOREMEDIATED COAL MINE EFFLUENTS USING CERTAIN BLOOD PARAMETERS OF FISH

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ABSTRACT

Toxicity analyses of the coal mine effluent (CME) on various blood parameters of catfish *Heteropneustes fossilis* exposed prior to, and after following phytoremediation individually with two macrophytes *Azolla pinnata* and *Lemna minor* were performed. Raw CME exposure cause decrease in total erythrocyte count (TEC) (75.13%), hemoglobin (Hb) (82.40%), hematocrit (Hct) (54.75%), mean corpuscular hemoglobin (MCH) (29.43%), mean corpuscular hemoglobin concentration (MCHC) (61.17%), O_2 carrying capacity (82.42%) and total protein concentration (72.39%) and increase in total leucocyte counts (TLC) (145.80%), glucose (73.89%), lipid (262.94%), cholesterol (110.50%) of fish blood. Further, fourfold increase in activities of lactate dehydrogenase (LDH), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and 2 to 3 time rise in the activities of superoxide dismutase (SOD), catalase (CAT) and lipid peroxidase (LPO) in the serum illustrate toxic manifestation of raw CME on fish tissues. Following exposure to *Lemna minor* and *Azolla pinnata* phytoremediated CMEs there were significant but incomplete improvements in most of the hematological and biochemical parameters of serum of fish. This could be probably due to higher amount of total accumulated metals (Metal Pollution Index) in the blood of raw CME exposed fish than in the fish exposed to either of the phytoremediated CMEs.

INTRODUCTION

Coal mine effluent (CME) is a heterogeneous mixture of several contaminants (e.g., heavy loads of several metals, chemical reagents, explosives, hardness, acidity, nitrate, sulphate, total dissolved solids, total suspended solids etc.) which pollutes the aquatic regime and threatens its aquatic flora and fauna (Grippo and Dunson, 1996; Adendorff, 1997; Martin and Black, 1998; Tiwary, 2001; Kennedy, 2002; Mylliemngap and Ramanujam, 2012; Khan *et al*., 2013). Amongst different xenobiotics, metals are however considered as one of the most detrimental pollutants due to their non-biodegradable nature and extensive toxicity to the life form especially when present in higher concentrations (Almeida *et al*., 2001; Almeida *et al*., 2005; De *et al*., 2010; Maceda-Veiga *et al*., 2012). Hence in this study, toxicity analyses of the CME generated at the Dudhichua mining project of Northern Coalfields Limited, Singrauli, India was undertaken applying fish bioassay. Fishes are directly/indirectly exposed to aquatic pollution and exposure to the ambient toxicants may disturb their physiology which gets reflected in their haematological parameters. Thus blood tissue of the economically important catfish *Heteropneustes fossilis* was subjected to hematological analysis. Phytoremediation technology for decontaminating metals of toxic effluents/wastewaters has now been widely used (Miretzky *et al*., 2004; Rai, 2008; Prasad and Singh, 2011). Therefore, the CME of Dudhichua was subjected to decontamination by phytoremediation using two locally available macrophytes *Lemna minor* (LPCME) and *Azolla pinnata* (APCME) separately. Bharti and Banerjee (2012) observed efficient removal of metals from CME by these plants but smaller amount of metals were still present in the phytoremediated CMEs thus the decontamination was partial. Several other authors have also demonstrated successful but incomplete detoxification of metals from variously contaminated waters/effluents applying phytoremediation method (Mishra *et al*., 2008; Rai, 2008). It is very likely that presence of remaining metals in the phytoremediated CME could continue to exert toxic stress to aquatic communities. However bioassay data to elucidate the effect of phytoremediated CME on aquatic life has rarely been addressed therefore efforts were also made to illustrate the level of toxic stress imparted by this partially detoxified CME on the fish using the identical hematological parameters. Being effective bioindicators of stressed conditions four important macromolecules (e.g., lipids, proteins, carbohydrates and nucleic acids) and activities of certain marker enzymes: lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase and antioxidative

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enzymes (superoxide dismutase, catalase and lipid peroxidase) were also analysed in the serum of fish.

MATERIALS AND METHODS

Site of effluent collection

The coal mine effluent (CME) was collected during July 2009 from the Dudhichua mining project at NCL, Singrauli, India in 50 L plastic tanks (pre-washed with 10% HNO_3).

Fish sampling

Irrespective of sex, healthy specimens of *H. fossilis* (50 ± 5.0 g and 17 ± 5.0 cm) were obtained from local fish pond at Chaukaghat, Varanasi. Fish were acclimatize in tap water for one month in laboratory and were fed with minced liver goat after every 24 hours prior to the renewal of the water and cleaning of the aquaria.

Sample analyses

Physicochemical properties and dissolved metal concentration in the raw CME, *Lemna minor* phytoremediated CME (LPCME), *Azolla pinnata* phytoremediated CME (APCME) and pond water were analysed by standard methods of the examination of water and wastewater as prescribed by American Public Health Association, American Water Works Association and Water Pollution Control Federation (APHA-AWWA-WPCF, 1998). For metal analyses (Fe, Mn, Ni, Zn, Cu, Pb, Cr and Cd) effluent samples were digested with HNO_3 and blood samples were digested with diacid (HNO_3 and $HClO_4$ in 2:1 ratio) on a hot plate maintained at 130° C. The dissolved metal concentrations (mg L^{-1}) in the effluent and blood samples were estimated using flame atomic absorption spectrophotometer (Perkin-Elmer Model 2380, Inc., Norwalk, CT, USA). The estimated detection limits of metals in the effluent (mg L^{-1}) and blood tissue (mg L^{-1}) are given in Table 1.

Table 1. Detection limits for metals

Metal	Fe	Mn	Zn	Cu	Pb	Cr	Cd	Ni
Detection limits in effluent (mg L^{-1})	0.08	0.06	0.05	0.001	0.01	0.002	0.0005	0.004
Detection limits in blood (mg L^{-1})	0.1	0.1	0.1	0.01	0.01	0.02	0.001	0.01

Experimental design

Three groups of ten fish each were exposed separately to 50 L of raw CME (having DO 5.56 ± 1.54 mg L^{-1}, pH 6.2 ± 0.56, water hardness 98.26 ± 0.045 mg L^{-1}, photoperiod 14L:10D); LPCME (having DO 5.78 mg L^{-1}, pH 6.9 ± 0.12, water hardness 85.5 ± 1.5 mg L^{-1}, photoperiod 14L:10D) and APCME (having DO 6.2 ± 0.5 mg L^{-1}, pH 7.4 ± 0.35, water hardness 90.2 ± 1.5 mg L^{-1}, photoperiod 14L:10D). One group of fish (n=10) were remained into 50 L of pond water (having dissolved oxygen (DO) of 6.5 mg l^{-1}, pH 7.2, water hardness 54 mg l^{-1}, photoperiod 14L:10D). Raw CME exposed fish were taken as control to quantify the levels of improvement obtained in various blood parameters of fish exposed to phytoremediated CME. The improvements in the blood profile of exposed fish were also compared to the wild fish (unexposed) to quantify the efficacy of phytoremediation. The effluents were not renewed however the essential criteria required for performing static bioassay were checked according to EPA (2002). The experiment was terminated after 26 days because beyond that fish in raw CME started dying gradually. The experiment was repeated thrice.

Hematological analysis

Blood samples from five fish from each group were taken out from the caudal vein after 12 and 26 days of exposure periods. Total Erythrocyte (TEC, RBC or red blood corpuscles) and leukocyte counts (TLC) were studied by Neubauer's improved hemocytometer using Hayem's and Tuerk's solutions respectively (Samuel, 1986). Hemoglobin (Hb) was measured using Sahli's hemoglobinometer. Hematocrit (Hct) values were determined by Wintrobe's methods. The oxygen-carrying capacity of the fish blood was calculated by multiplying the Hb content by 1.25 oxygen combining power of Hb/g (Johansen, 1970). Mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) were calculated by standard formulae given by Dacie and Lewis (1991).

$$MCHC\ (g/dl) = \frac{Hb}{Hct} \times 100$$

$$MCH\ (pg) = \frac{Hb}{RBCs} \times 10$$

$$MCV\ (mm^3) = \frac{Hct}{RBCs} \times 10$$

Metal pollution index

Metal pollution index (MPI) was calculated using the equation proposed by Usero *et al*. (1997) to compare the total metal accumulation load in blood of different groups of fish:

$$MPI = (C_{f1} \times C_{f2} \times C_{fn})^{1/n}$$

Where, C_{fn} is the concentration for the metal n in the sample.

Biochemical analysis

For biochemical analyses of serum, blood was centrifuged at 3000 rpm for 10 min. Concentrations of total proteins, glucose, lipid, cholesterol and activities of lactate dehydrogenase (LDH), alkaline phosphatase (ALP), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidase (LPO) were estimated in serum by the methods of Lowry *et al*. (1951), Schmidt (1961), Folch and Stanley (1957), Abell *et al*. (1952), Wroblewskil and Ladue (1955), Tennis Wood *et al*. (1976),

Das *et al*. (2000), Aebi (1984) and Ohkawa *et al*. (1979) respectively. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were determined following Bergmeyer *et al*. (1985) method.

Statistical analyses

All data were expressed as mean ± standard error of means (SEM). Data were analysed by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test (DMRT) to find out significant difference at the level of $p < 0.05$ between different groups of fish.

RESULTS AND DISCUSSION

Analyses of physicochemical properties of raw CME exhibited toxic nature of the effluent due to contamination with heavy loads of metals and several other harmful xenobiotics (Bharti and Banerjee, 2012). They demonstrated that phytoremediation by either of the macrophytes *L. minor* and *A. pinnata* have significantly improved the dissolved oxygen (DO) content and decreased the metal load of CME however the detoxification was partial (Bharti and Banerjee, 2012) (Table 2). Discharge of these incompletely detoxified effluents into the aquatic ecosystem would certainly increase the metal contaminants of the wetlands (Barber *et al*., 2011).

Fe > Mn > Zn > Cu > Cr > Pb > Ni > Cd after 26 days (Table 2). In either of the phytoremediated CMEs exposed fish, the concentration of these metals in blood got significantly ($p < 0.05$) lowered (Table 2). The percent decrease in metal concentration after 26 days in the of blood of LPCME exposed fish were: 50.12% of Fe, 52.34% of Mn, 53.06% of Zn, 63.67% of Cu, 44.77% of Ni, 75.54% of Cr and in APCME exposed fish were: 47.95% of Fe, 50.54% of Mn, 50.31% of Zn, 53.88% of Cu, 44.38% of Ni and 93.61% of Cr compared to raw CME exposed fish (Table 2). Concentration of Pb and Cd were below the detection levels (BDL) in LPCME and APCME exposed fish while Cr was detected after 26 days in only LPCME exposed fish (Table 2).

This could possibly be due to the presence of metals left in the effluent even after phytoremediation. Metal from the aquatic media enter into the blood of fish via absorption by gills and skin of the fish (Banerjee, 2007). Continued exposure to even phytoremediated CMEs has lead to metal accumulation in fish blood (Table 2). It was also noticed that the amount of metals absorbed in the blood were directly proportional to the periods of exposure. Barber *et al*. (2011) also observed that aquatic animals accumulated higher/lethal concentrations of dissolved metals over long periods from extremely low metal concentrations in water. 'Polluted' from 'non-polluted' ecosystem can be easily distinguishes by the metal pollution

Table 2. Accumulation of metal (mg L^{-1}) in blood of fish exposed to raw CME (control), LPCME and APCME and the MPI values after days (d) compared to wild fish

	Raw CME (Bharti and Banerjee 2012)	Raw CME exposed (Control)		LPCME[$] (Bharti and Banerjee 2012)	LPCME[@] exposed		APCME (Bharti and Banerjee 2012)	APCME[*] exposed		Pond water	Wild fish (Unexposed)
Metals		12 d	26 d		12 d	26 d		12 d	26 d		(Average value)
Fe	22.906± 0.023	317.32±21.05^{b}	325.65±31.15^{b}	1.586 ± 0.05	158.77±2.56^{c}	162.46 ± 5.09^{c}	1.05 ± 0.05	160.0 ±15.75^{c}	169.57 ± 25.53^{c}	0.04 ± 0.00	113.87 ± 13.15^{a}
Mn	9.606 ± 1.599	25.98 ± 2.11^{b}	40.43 ± 2.75^{c}	0.047 ± 0.0	15.07 ± 1.09^{d}	19.27 ±1.28^{d}	0.19 ± 0.09	18.12 ± 0.67^{d}	20.0 ± 1.51^{d}	BDL	9.02 ± 3.53^{a}
Zn	1.034 ± 0.15	27.01 ± 3.04^{b}	32.36 ± 4.12^{b}	0.034 ± 0.005	11.95 ± 0.25^{c}	15.19 ± 1.17^{c}	0.05 ±0.00	12.03 ± 2.77^{c}	16.08 ±1.35^{c}	0.3 ± 0.00	8.09 ± 1.09^{a}
Cu	2.039 ± 0.231	12.17 ± 0.58^{b}	22.18 ± 3.08^{c}	0.023 ± 0.003	1.01 ±0.08^{d}	8.06 ± 0.09^{a}	0.14 ± 0.01	1.11 ± 0.02^{d}	10.23 ± 0.007^{b}	0.07 ±0.00	5.54 ±0.52^{a}
Ni	0.856 ± 0.187	3.87 ± 0.09^{b}	5.16 ± 1.50^{b}	0.047 ± 0.01	2.62 ± 1.7^{b}	2.85 ± 0.25^{b}	0.29 ± 0.01	2.71 ± 0.21^{b}	2.87 ± 0.02^{b}	BDL	0.57 ± 0.03^{a}
Pb	0.669 ± 0.105	11.89 ± 0.002^{b}	16.07 ± 2.15^{b}	0.11 ± 0.008	^ BDL^{a}	BDL^{a}	0.09 ± 0.01	BDL^{a}	BDL^{a}	BDL	BDL^{a}
Cr	0.182 ± 0.007	12.06 ± 1.92^{b}	16.27 ± 1.52^{b}	0.043 ± 0.002	BDL^{a}	3.93 ± 0.05^{a}	0.04 ± 0.01	1.06 ± 0.04^{a}	3.04 ± 0.05^{a}	BDL	5.66 ± 1.52^{b}
Cd	0.0598 ±0.008	1.36 ± 0.06^{b}	2.17 ± 0.54^{b}	0.008 ± 0.001	BDL^{a}	BDL^{a}	0.009 ± 0.001	BDL^{a}	BDL^{a}	BDL^{a}	BDL^{a}
#MPI		**14.583**	**20.194**		**9.457**	**12.619**		**6.881**	**12.885**		**7.219**

All values were expressed as mean ± standard error of means (SEM) (n=15), [@]LPCME- *L. minor* phytoremediated coal mine effluent,
[*]APCME- *A. pinnata* phytoremediated coal mine effluent,
[#]MPI- Metal pollution Index, [^]BDL- Below detectable limit (here considered as 0.00 for statistical analyses)
[a-d] Different letters in a row indicate significant difference between the groups according to DMRT ($p < 0.05$) and identical letter denotes insignificant difference them.

Concentration of metals in blood

Concentration (mg L^{-1}) of eight metals in the blood of fish after exposure of 12 and 26 days to raw CME, LPCME and APCME has been summarized in Table 2. The concentration of metals in blood of raw CME exposed fish were in the order

index (MPI). Its value above 1 indicates elevated levels of the metals in the system and consequently regarded as 'polluted' (Teodorovic *et al*., 2000). Keeping this in mind we have also investigated MPI index for the blood of fish exposed to all the three different type of CMEs (raw CME, LPCME and APCME) and it was found that the MPI value was highest for fish exposed to raw CME followed by LPCME and APCME

(Table 2). Lowered MPI values of fish exposed to phytoremediated CMEs indicated their successful decontamination by phytoremediation. However increased MPI value (< 1) of wild (unexposed) fish indicate great danger of damage to fish due to metal pollution of wetlands (Table 2).

Hematological and biochemical parameters

Raw CME exposure severely affected the hematological parameters (Table 3). The values of TEC, Hb, Hct, MCH, MCHC, O_2 carrying capacity of blood decreased significantly ($p < 0.05$) while TLC, MCV increased on both the periods of raw CME exposure (Table 3). Decrease in these blood parameters reflects the immediate response of the organism negotiating the stressed condition (Nussey *et al*., 2002; Wabhi *et al*., 2004; Kumar *et al*., 2005; Kori-Siakpere and Obogu, 2008; Kori-Siakpere *et al*., 2010; Firat *et al*., 2011). Goel and Gupta (1985) reported significant decrease in TEC, Hb and Hct of *H. fossilis* exposed to sublethal concentration of Zn. Pollutants such as metals, pesticides, insecticides etc. have also been reported to cause considerable reduction in TEC, Hb and Hct of fish (Nanda *et al*., 2010; Mekkawy *et al*., 2011; Thenmozhi *et al*., 2011; Adakole, 2012; Vani *et al*., 2012). Hence metal and other contaminants in raw CME might have caused decrease in TEC and Hb contents of exposed fish (Table 3). The other reason for the lowered RBC levels of raw CME exposed fish might be due to suppression of haemopoeitic activity of head kidney, apart from its role of increased elimination of dysfunctional RBC which consequently have caused decline in Hb content (Stormer *et al*., 1996; Sawhney and Johal, 2000). The decrease in Hb might also be because of either rise in their rate of destruction or reduction in their biosynthesis rate (Vani *et al*., 2012). According to Kori-Siakpere *et al*. (2010) lowered Hb and Hct is perhaps due to hemodilution resulting from impaired osmoregulation via the gill epithelium to lowers the levels of pollutants in the blood vascular systems (Wedemeyer *et al*., 1999). The TLC value increased significantly in raw CME exposed fish while decreased in the blood of fish exposed to either of the phytoremediated CMEs indicating less severe damage to their tissues (Table 3).

Increased TLC generally occurs as an immunological response in organisms to counter a variety of stressed conditions (Devis *et al*., 2008; Nanda *et al*., 2010). Adeyemo (2005) and Gabriel *et al*. (2007) respectively recorded significantly higher values of TLC of *Clarius gariepinus* exposed to cassava mill effluent and refined petroleum oil (kerosene). The erythrocyte indices such a MCV, MCH, MCHC have a particular importance in diagnosis for the type of anemia in large number of animals hence applied (Coles, 1986). Decreased RBC and Hb concentration along with significant increase in MCV values in the blood of raw CME exposed fish suggest increase in erythrocytes volume (Table 4). The higher values of MCV and lowered MCH, MCHC and O_2 carrying capacity of blood of fish after 26 days of raw CME exposure confirm occurrence of macrocytic type of anemia (Santhakumar *et al*., 1999; Moharram *et al*., 2011) (Table 4). Phytoremediation with both the macrophytes improved the levels of contamination of the CME as manifested by improved values of TEC, Hb, Hct, MCV in blood of the fish exposed to both of the phytoremediated CMEs even though their levels failed to reach to the those of the levels of the wild fish (unexposed) (Table 3 and 4). It was interesting to note that the hematological profile of fish exposed to LPCME and APCME improved after

Table 3. Haematological parameters of the blood of fish exposed to raw CME and phytoremediated CME after 12 and 26 days (d) compared to wild fish (unexposed)

	TEC ($\times 10^6$ /mm^3)		TLC ($\times 10^3$ /mm^3)		Hb (g dL^{-1})		Hct (%)	
	12 d	26 d	12 d	26 d	12 d	26 d	12 d	26 d
Wild fish (Unexposed)	4.06±0.02^a	4.1±0.06^a	4.08±0.08^a	4.17±0.06^a	12.2 ± 0.02^a	12.1 ± 0.03^a	18.87±0.15^a	19.51±0.2^a
Raw CME exposed (Control)	1.29±0.01^b (–68.23)	1.02±0.02^b (–75.13)	9.1±0.05^b (+123.03)	10.25±0.07^b (+145.80)	4.97 ± 0.34^b (–59.3)	2.13 ± 0.05^b (–82.4)	12.6±0.4^b (–33.23)	8.83±0.61^b (–54.75)
LPCME exposed	3.01±0.05^c (–25.86)	3.73±0.03^a (–9.03)	5.25±0.02^c (+28.63)	8.91±0.04^b (+86.75)	9.40 ± 0.27^c (–22.95)	8.97 ± 0.01^c (–25.87)	20.25±0.5^a (–7.12)	16.39±0.9^a (–10.08)
APCME exposed	2.45±0.01^c (–39.66)	3.00±0.03^d (–26.83)	6.81±0.08^d (+66.84)	8.34±0.18^b (+74.8)	9.20±0.21^c (–24.60)	7.83 ± 0.03^c (–35.29)	18.45±0.32^a (–2.4)	16.12±0.16^a (–17.47)

All values were expressed as mean ± SEM (n=15)
Values in parentheses are in percentage calculated by assuming value of parameter of wild fish as 100%.
(+) indicate increase and (–) indicate decrease
$^{a-d}$ Different letters in column indicates significant difference ($p < 0.05$) between them according to DMRT. Identical letter denotes insignifi difference between them.

Table 4. Erythrocyte indices of fish exposed to raw and phytoremediated CMEs after 12 and 26 days (d) of exposure periods compared to wild fish (unexposed)

	MCV (mm^3)		MCH (pg)		MCHC (g dL^{-1})		O_2 carrying capacity of blood (ml $0_2/g^{-1}$/Hb)	
	12 d	26 d	12 d	26 d	12 d	26 d	12 d	26 d
Wild fish (Unexposed)	46.48 ± 0.2[a]	47.58 ± 0.1[a]	30.05 ± 0.3[a]	29.5 ± 0.5[a]	64.65 ± 0.22[a]	62.0 ± 0.01[a]	15.25 ± 0.02[a]	15.13 ± 0.03[a]
Raw CME exposed (Control)	97.67 ± 0.11[b] (+109.99)	86.57 ± 0.23[b] (+81.79)	38.52 ± 0.05 (+28.27)	20.88 ± 0.01[b] (−29.43)	39.44 ± 0.31[b] (−39.26)	24.12 ± .13[b] (−61.17)	6.21 ± 0.34[b] (−59.28)	2.66 ± 0.05[b] (−82.42)
LPCME exposed	67.27 ± 0.42[c] (+44.65)	43.94 ± 0.24[c] (−7.65)	31.23 ± 0.52[a] (+3.99)	24.04 ± 0.2[a] (−18.51)	46.41 ± 0.08[b] (−28.21)	54.72 ± 0.25[a] (−11.74)	11.75 ± 0.27[c] (−22.95)	11.21 ± 0.01[c] (−25.91)
APCME exposed	75.31 ± 0.34[d] (+61.92)	53.73 ± 0.41[d] (+12.92)	37.55 ± 0.17[a] (+24.96)	26.10 ± 1.6[a] (−11.53)	49.86 ± 0.39[b] (−22.88)	48.57 ± 1.2[c] (−21.66)	11.50 ± 0.21[c] (−24.59)	9.79 ± 0.03[c] (−35.29)

All values were expressed as mean ± SEM (n=15)
Values in parentheses are in percentage calculated by assuming value of parameter of wild fish as 100%.
(+) indicate increase and (–) indicate decrease
[a-d] Different letters in column indicates significant difference ($p < 0.05$) between them according to DMRT. Identical letter denotes insig difference between them.

Table 5. Biochemical parameters of serum in fish exposed to raw and phytoremediated CMEs after 12 and 26 days (d) of exposure periods compared to wild fish (unexposed)

	Total glucose (mg dL^{-1})		Total cholesterol (mg dL^{-1})		Total lipid (mg dL^{-1})		Total protein (mg dL^{-1})	
	12 d	26 d	12 d	26 d	12 d	26 d	12d	26d
Wild fish (Unexposed)	78.86 ± 4.44[a]	79.43 ± 3.5[a]	269.27 ± 4.1[a]	255.81 ± 5.7[a]	231.66 ± 16.16[a]	138.04 ± 13.42[a]	8.6 ± 0.12[a]	8.2 ± 0.27[a]
Raw CME exposed (Control)	138.53 ± 4.97[b] (+61.74)	167.06 ± 3.6[b] (+73.89)	325.91 ± 5.6[a] (+74.55)	456.82 ± 3.2[b] (+110.50)	511.0 ± 15.69[b] (+120.58)	501.0 ± 14.5[b] (+262.94)	3.29 ± 0.01[b] (−54.59)	2.14 ± 0.03[b] (−72.39)
LPCME exposed	67.98 ± 5.81[a] (−16.73)	59.33 ± 3.25[c] (−35.58)	178.23 ± 2.4[b] (−14.35)	222.12 ± 7.4[a] (−25.25)	428.3 ± 35.27[a] (+84.85)	382.9 ± 32.07[c] (+177.22)	7.16 ± 0.04[c] (−16.05)	5.28 ± 0.053[c] (−23.42)
APCME exposed	62.57 ± 2.7[a] (−20.68)	58.63 ± 2.5[c] (−39.61)	172.44 ± 5.8[b] (−21.16)	202.51 ± 13.28[a] (−26.13)	360.51 ± 15.9[c] (+55.60)	308.2 ± 11.32[d] (+123.14)	6.82 ± 0.023[c] (−29.34)	4.95 ± 0.042[c] (−38.36)

All values were expressed as mean ± SEM (n=15)
Values in parentheses are in percentage calculated by assuming value of parameter of wild fish as 100%.
(+) indicate increase and (–) indicate decrease
[a-d] Different letters in column indicates significant difference ($p < 0.05$) between them according to DMRT. Identical alphabet denotes in difference between them.

exposure of 12 days however prolonged exposure of 26 days to either of the phytoremediated CMEs caused decline in the improvements (Table 3 and 4). The toxicants present in the aquatic ecosystem exert great stress to the fish. To negotiate this stress, there is extensive mobilization of biomolecules which disturb the equilibrium existing between them and thus results in significant alteration in the biochemical compositions of the tissues. Therefore measurement of serum biochemical parameters such as concentration of protein, glucose, lipids and cholesterol are successfully being applied to monitor the stressed condition of the fish and damaged condition of organs caused by the toxicants (Das *et al*., 2003; Nanda *et al*., 2010; Firat and Kargan, 2010; Vani *et al*., 2012). In our study, following raw CME exposure, excepting total proteins, concentrations of glucose, lipids and cholesterol increased greatly in fish blood (Table 5). Significant hyperglycemia in the blood of *Oreochromis mossambicus* exposed to metals (Cu and Pb) has also been observed by Firat *et al*. (2011). Decreased concentration of protein and increased amount of glucose in blood of fish exposed to raw CME (Table 5) might be due to proteolysis to produce extra glucose molecules via gluconeogenesis or due to breakdown of glycogen to meet the higher energy demand to combat the toxic stress of the raw CME. Recently, Bharti and Banerjee (2013) have shown significant decline in total glycogen concentration of different organ systems of raw CME exposed fish. The hyperglycemic response indicates disturb carbohydrate metabolism, possibly caused by enhanced glucose 6-phosphatase activity in the liver and synthesis of glucose from extrahepatic tissue proteins and amino acids (Almeida *et al*., 2005; Yousef *et al*., 2008; Palaniappan and Vijayasundarum, 2009). The higher levels of lipids and cholesterol in the blood of raw CME exposed fish might be due to their release from the cell membranes of damaged tissue cause by the CME exposure (Table 5).

Generally, cholesterol concentrations in the serum of metal-exposed fish decrease (Kaur and Kaur, 2006). Increased concentration of these moieties in the blood of raw CME exposed fish might have been contributed by the decreased lipid concentration of vital tissue systems of raw CME exposed fish as suggested by Bharti and Banerjee (2013). Allen *et al*. (2005) reported that the increased concentration of cholesterol could be due to disfunctioning of the liver leading to disorder of lipid and lipoprotein metabolism in hepatocytes. Following exposure to either of the phytoremediated CMEs the total protein concentration of fish blood improved significantly but their levels still continued to be below the levels of the wild fish (unexposed) (Table 5). The concentration of total glucose, lipids and cholesterol of blood of fish exposed to either of the phytoremediated CMEs however improved significantly (Table 5). Assay of activities of certain marker enzyme (e.g., ALP, ALT, AST and LDH,) in serum of fish exposed to raw CME, LPCME and APCME were also performed (Fig. 1a, 1b, 1c and 1d). Significantly increased activities of ALP, ALT, AST and LDH in the blood of fish exposed to raw CME indicate pathological condition of fish (Fig. 1a, 1b, 1c and 1d). In both the phytoremediated CMEs the activities of the marker enzymes decreased significantly but still continued to be above the levels of wild fish (unexposed) (Fig. 1a, 1b, 1c and 1d). Firat and kargan (2010) and Firat *et al*. (2011) illustrated elevated values of AST and ALT in blood of Nile Tilapia *Oreochromis niloticus* exposed to Zn and Cd and Cu and Pb respectively.

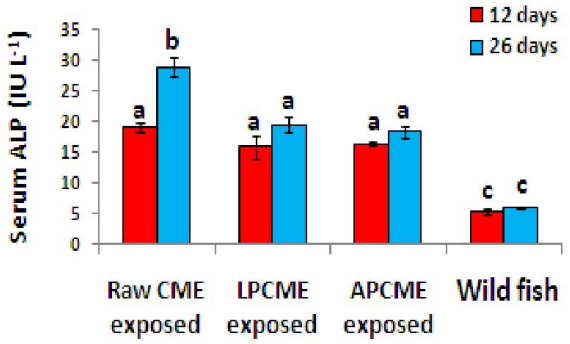

Fig. 1a ALP activity in fish blood

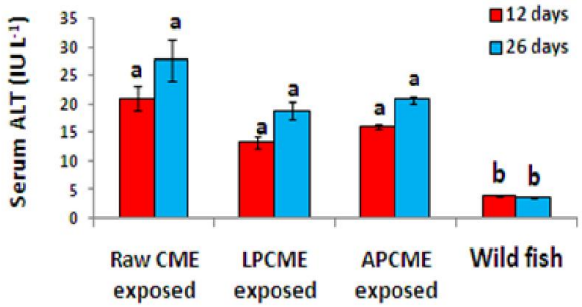

Fig. 1b ALT activity in fish blood

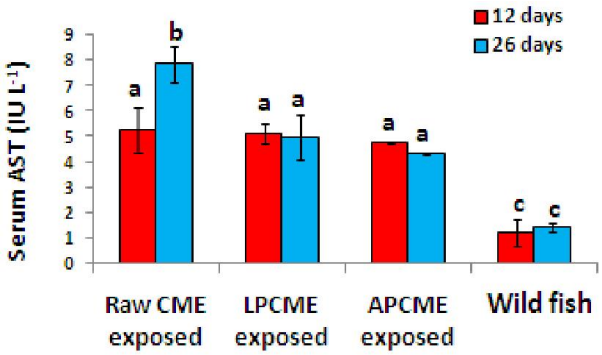

Fig. 1c AST activity in fish blood

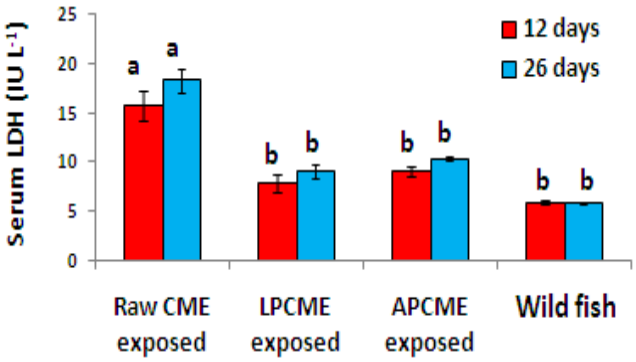

Fig. 1d LDH activity in fish blood

Increased LDH activities indicate towards compensatory increase in cellular metabolism and thereby more production of lactic acid causing acidosis. According to Heath (1995) chronic exposures to low pH leads to reduction in O_2 flow to the blood and enhancement in enzymatic activities of fish which are also in line with our findings. Also, increased activities of ALT, AST, ALP, and LDH indicate degeneration changes and hypofunction of liver, especially liver disease. Increased activities of both the transaminase (AST and ALT) in exposed *H. fossilis* serum could enhance the production of amino acids which via the tricaboxylic acid cycle could generate extra glucose moieties to meet the additional demand of energy during stressed condition (Almeida *et al*., 1995; Vaglio and Landriscina, 1999; Kori-Siakpere *et al*., 2010) Metals and other xenobiotics are known to cause significant increase in production of reactive oxygen species (ROS) leading to "oxidative stress" resulting in tissue damage and various dysfunctions in lipid, protein and DNA metabolism (Wilhelm Filho *et al*., 2000; Ercal *et al*., 2001). Due to the inhibitory effects of the antioxidant enzymes on oxy-radical formation, SOD-CAT system is known as the first line of defense against oxidative stress (Soimasuo *et al*., 1995; Atli and Canli, 2010; Nanda *et al*., 2010). Raw CME exposure led to increased activities of both these inducible enzymes in the serum of fish (Fig. 2a and 2b) to protect the tissue damage caused due to high loads of metals and other xenobiotics of the effluent. Accumulation of the environmental pollutants and metals such as Cu, Pb, Cr and Cd has been shown to cause alterations in the activities of these enzymes (Almeida *et al*., 1995; Atli and Canli, 2010; Firat *et al*., 2011).

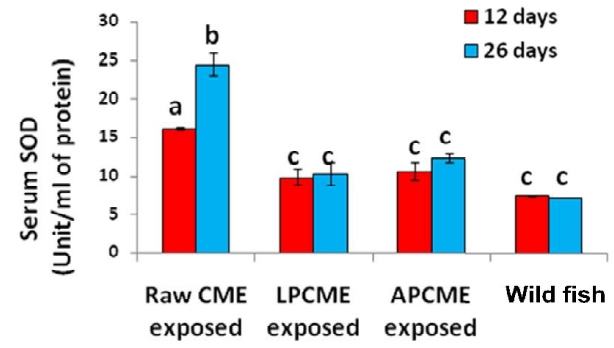

Fig. 2a SOD activity in fish blood

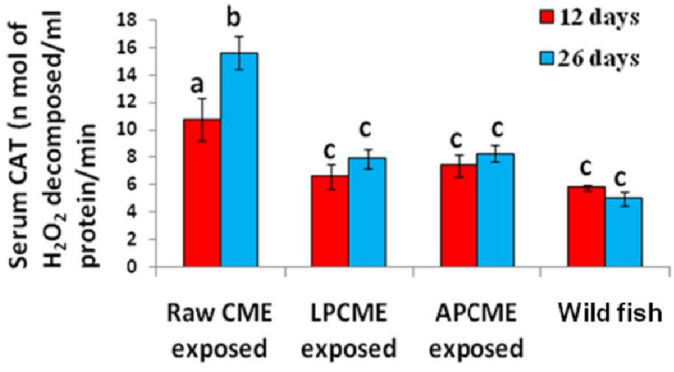

Fig. 2b Catalase activity in fish blood

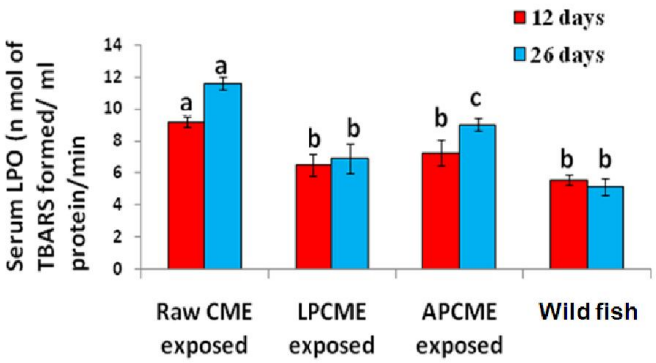

Fig. 2c LPO activity in fish blood

Enhanced activity of LPO in raw CME exposed fish also indicates the ROS-induced peroxidation leading to the destruction of cell membranes (Fig. 2c). According to Atli and Canli (2010) the activities of the enzymes depend on the redox state of the metals. Improvement in the activities of all these enzymes in the blood of fish exposed to either of the decontaminated CMEs by phytoremediation indicates towards less damaged condition of fish tissue systems which might be due to less absorption of metals by their tissues. However levels of enzymatic activities never reached to those of the levels of the wild fish which designate incomplete decontamination of the effluent even after phytoremediation.

Conclusion

Raw CME exposure severely altered the blood profile of fish indicating its devastating effect on fish health. Exposure to decontaminated CMEs phytoremediation significantly improved the hematological and biochemical constituents of fish blood but their levels failed to reach to the levels of control (wild) fish. This is because of metals were still present in the phytoremediated CMEs even though in smaller quantities and fish continued to absorb the metal and other pollutants from these decontaminated effluent on prolonged exposure. Thus, phytoremediated CMEs need further purification prior to their disposal into the aquatic ecosystem.

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