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RESEARCH ARTICLE

BIOASSAY ANALYSIS OF EFFICACY OF PHYTOREMEDIATION TECHNOLOGY IN DECONTAMINATING THE TOXIC EFFLUENT RELEASED DURING RECOVERY OF METALS FROM POLYMETALLIC SEA NODULES

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ABSTRACT

Efforts have been made to analyse the efficacy of decontamination by phytoremediation technology using fish bioassay method. For this purpose Fish (*L. rohita*) were exposed to raw effluent, *Lemna minor* phytoremediated & *Azolla pinnata* phytoremediated effluent separately for a period of 20 days. Heavy metal analysis, different haematological and biochemical parametres were studied in the blood of raw effluent exposed and phytoremediated effluent exposed fish. Concentration of metals in the blood of the fish exposed to phytoremediated effluents was found to be less than those exposed to raw PMN effluent, perhaps due to decreased concentration of the metals in both the phytoremediated effluents. Different blood profiles like TEC, TLC, Hb, Ht, MCV, MCH, MCHC and differential leukocyte count also improved in phytoremediated effluent exposed fish when compared to raw effluent exposed ones. Concentration of proteins, lipids, glucose, cholesterol & activities of certain enzymes like AST, ALT, ALP, SOD, catalase, lipid peroxidase and lactate dehydrogenase of the blood also showed significant improvement due to phytoremediation of the effluent. Hence exposure to the phytoremediated effluents caused less damage in the fish in comparison to those exposed to raw effluent. However improvement in the health of phytoremediated effluent exposed fish did not reach to the levels of control wild fish.

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INTRODUCTION

Various domestic, industrial and mining toxic effluents are detoxified by several chemical, physical and mechanical processes for decontamination prior to their release into the aquatic ecosystem. Such types of detoxifying processes are not enough to contain pollution. Often due to cost factor there is a tendency to evade the mandatory treatment of the effluent for detoxification by the industrial houses. Hence biologists intervened and tried to evolve cheap and effective methods of phytoremediation for decontamination of the toxic effluents polluted with heavy metals (Mishra *et al*., 2008; Rai and Tripathi, 2009; Vaseem and Banerjee, 2012). Due to acute scarcity of certain economically useful metals like Cu, Cr, Co, Mn and Ni, etc., metallurgist at National Metallurgical Laboratory, Jamshedpur, India, are trying to develop indigenous technique to extract metals from polymetallic sea nodules which are abundantly found at sea bed.

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For the metal extraction purpose the nodules are processed through many metallurgical methods including reduction, ammonia leaching, solvent extraction, electro winning and smelting (Kumar *et al*., 1990; Jana *et al*., 1999; Agarwal and Goodrich, 2008; Biswas *et al*., 2009). Such metallurgical processes also generate large amount of toxic effluent (PMN effluent). The toxicity of this waste water is due to contamination of toxic metals which cause extensive damage to the aquatic fauna (Vaseem and Banerjee, 2013a, b). To lower the toxicity load of metals in this effluent, Vaseem and Banerjee (2012) successfully demonstrated decontamination of the effluent by phytoremediation technology. There are numbers of reports related to application of phytoremediation in decontaminating the effluent released from various industries (Rai, 2008, 2010; Rai and Tripathi, 2009). However, there are no bioassay data using fish to analyse the improvement in effluent quality following phytoremediation. Hence to bridge this gap the decontaminated effluents were subjected to fish bioassay analysis using a major carp (*Labeo rohita*) as bioindicator. For this purpose fish (*Labeo rohita*) were exposed to raw effluent, *Azolla*-phytoremediated effluent (APE) and *Lemna*-phytoremediated effluents (LPE).

Metals accumulation in the blood (Mn, Fe, Cu, Cr, Pb, Zn & Ni) and different blood profiles like total erythrocyte count (TEC), total leukocyte count (TLC), haemoglobin (Hb) & haematocrit (Ht), certain calculated indices (Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), oxygen carrying capacity of the blood), differential leukocyte count, different biochemical properties of the blood (concentration of proteins, glucose & cholesterol) and various enzyme activities like aspartate amino transferase (AST), alanine amino transferase (ALT) , alkaline phosphatase (ALP), superoxide dismutase (SOD), catalase and lactate dehydrogenase (LDH) were analysed in raw effluent exposed as well as decontaminated effluents i.e. *Azolla* phytoremediated effluent exposed and *Lemna* phytoremediated effluent exposed fish.

MATERIALS AND METHODS

Analysis of PMN effluent and its phytoremediation

Physicochemical analyses and concentrations of different metals (Fe, Mn, Zn, Cu, Pb, Cr and Cd) in PMN effluent were studied following the standard methods for examination of water and waste water (APHA 1998) and atomic absorption spectrophotometer (AAS) respectively (Perkin-Elmer Model 2380, Inc., Norwalk, CT, USA). The details of the physicochemical characteristics of raw PMN effluent have already been described and the effluent was found to be extremely toxic (Vaseem and Banerjee, 2012a). When phytoremediated with two different macrophytes (*Azolla pinnata* and *Lemna minor*) for 7 days separately, the effluent got partially but significantly detoxified as manifested by decreased concentration of the different metal species (Vaseem and Banerjee, 2012b). The percentage of decontamination of the metals reported after phytoremediation were in the order: 96 % of Mn, 97% of Cu, 70% of Fe, 96% of Pb, 93% of Cr and 78% of Cd by *Azolla* and 94% of Mn, 86% of Cu, 62% of Zn, 74% of Fe, 84% of Pb, 63% of Cr and 78% of Cd by *Lemna* phytoremediation.

Experimental Fish

Healthy specimens of the Indian major carp *L. rohita,* were collected from the hatchery situated in Banaras Hindu University, Varanasi, India. The fish were acclimated to laboratory conditions for one month in plastic tanks equipped with a continuous supply of well-aerated and dechlorinated water at 24 ± 2^{o}C and under natural photoperiod. During this period, fish were fed ad libitum with commercial fish pellets (40% of protein). The water was renewed after every 24 hrs with regular cleaning of the tanks. The water quality parameters were: dissolved oxygen (7.0–7.5 mg L^{-1}), pH (7.1–7.4), conductivity (125–130 µS cm^{-1}), alkalinity (35–43 mg L^{-1} as $CaCO_3$) and total hardness (39–50 mg L^{-1} as $CaCO_3$).

Experimental design

Acclimated fish were divided into four groups of 20 fish: one group exposed to 50 L of tap water; second group exposed to 50 L of the raw PMN effluent (BOD: 182 ± 3 mgL^{-1}, pH: 5.2, Sodium: 130.26 ± 1.96 mgL^{-1}, Potassium: 3.42 ± 0.07 mgL^{-1}, Sulphate: 2300 ± 4.515 mgL^{-1}, Carbonate: 296 ± 2.2 mgL^{-1}, Mn 4.9 mgL^{-1}, Cu 1.432 mgL^{-1}, Zn 0.816 mgL^{-1}, Fe 0.762 mgL^{-1}, Pb 0.655 mgL^{-1}, Cr 0.07 mgL^{-1}, Cd 0.018 mgL^{-1}); third to 50L of APE and the fourth to 50 L of LPE in a 80 L capacity of plastic tubs for maximum period of 20 days (beyond which fish did not survive) with regular renewal of water after every 5 days of interval (semi-static bioassay). Fish were regularly fed during experiment. Eight fish from each group were cold anesthetized and sacrificed after 20 days of exposure. Three replicates of each experimental set were prepared. Blood from cold anaesthetised fish was collected from the cardiac region by puncturing the heart using a plastic disposable syringe fitted with a 26-gauge needle, moisturised with heparin and was immediately transferred to separate heparinised chilled plastic vials and immediately returned to the ice box.

Haematological studies

Haemoglobin was estimated by using the Sahli's hemoglobinometer. Oxygen carrying capacity of the blood was detected by multiplying the haemoglobin content by 1.25 oxygen combining power of Hb/g (Johansen, 1970) . Both the leukocyte counts and Erythrocyte were studied by Neubauer's improved hemocytometer using Tuerk's and Hayem's solution respectively as a diluting fluid (Samuel, 1986). Hematocrit values were estimated by Wintrobe's methods. Differential leukocytes count (DLC) was carried out by preparing a thin blood smear and staining it with Geimsa stain. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated by the standard formulae Dacie & Lewis (1991).

$$MCV = \frac{\text{PCV/1,000 ml blood}}{\text{RBC in millions/mm3}} = \text{fl}$$

$$MCH = \frac{\text{Hb in g/1,000 ml blood}}{\text{RBC in millions/mm3}} = \text{pg}$$

$$MCHC = \frac{\text{Hb in g/100 ml blood}}{\text{PCV/100 ml}} \times 100 = \text{g/dl}$$

Metal analyses

The metal concentrations of blood sample was analysed following standard methods for the examination of water and wastewater as prescribed by American Public Health Association, American Water Works Association and Water Pollution Control Federation (APHA, 1998). For metal analysis, the blood samples were digested with diacid (HNO3 and HClO4 in 2:1 ratio) on a hot plate maintained at 130 °C. The dissolved metal concentrations in the blood samples were analysed using a flame atomic absorption spectrophotometer (Perkin-Elmer Model 2380, Inc., Norwalk, CT, USA). The results were expressed as milligrams per litre. Detection limits for metal ions in milligrams per litre were 0.08 for Fe, 0.06 for Mn, 0.05 for Zn, 0.001 for Cu, 0.01 for Pb, 0.002 for Cr and 0.004 for Ni.

Biochemical analyses

For biochemical analyses of the serum, the blood (unheparinised) was subjected to centrifugation for 15 minutes at 3000 rpm. The concentrations of glucose, cholesterol, protein, lipid and activities of transaminases (AST & ALT) and ALP in the serum were estimated by the methods of Seifter *et al.* (1950), Zlatkis *et al.* (1953), Lowry *et al.* (1951), Reitman &Franckel (1957) and Bregmayer (1957) respectively. The activity of LDH was measured by the method of Wroblewski and Ladue (1955). The activity of SOD, catalase and lipid peroxidise were determined by the method of Das *et al*. (2000), Aebi (1984) and Ohkawa (1979) respectively.

Statistical analyses

For statistical analyses one-way analysis of variance (ANOVA) ($p< 0.05$) was performed followed by Duncan's Multiple Range Test (DMRT). Values in the tables and figures are given in mean ± SD. In tables and figures alphabets denote the result of DMRT. Different alphabets show significant difference ($p < 0.05$) in the different values of wild control fish, raw effluent exposed fish, APE exposed fish and LPE exposed fish.

RESULTS AND DISCUSSION

Following exposure to raw PMN effluent, toxicity induced extensive alterations in the various haematological parameters of *L. rohita* were noticed (Table 1). Exposure to the raw effluent caused significant accumulation of different metals in the blood of fish (Figure 1).

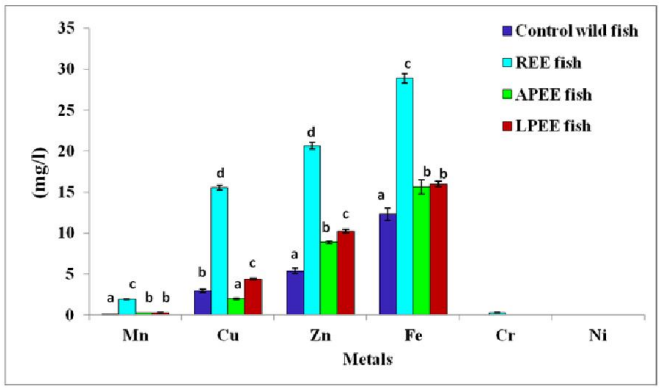

Figure 1. Metal accumulation in the fish exposed to phytoremediated effluent & its comparison to those of raw effluent exposed ones and wild controls

This might be the cause for significant alterations in the different haematological properties of raw effluent exposed fish (Table 1). Accumulation of metals in various tissues like muscles, liver, gills, kidney, brain and skin was also noticed in the fish exposed to raw PMN effluent (Vaseem and Banrejee, 2013). When fish were exposed to the phytoremediated effluents, there was significant lesser accumulation ($p<.05$) of different metals in the blood tissue (Figure 1) which showed the active decontamination of the metals by macrophytes. However metals accumulation in the blood failed to reach the levels of control ones. This might be due to incomplete decontamination of the effluents by both the plants. There were also lesser damage ($p<0.05$) in all the different haematological properties e.g. TEC, TLC, Hb, Ht, MCV, MCH, MCHC (Table 1) & differential leukocytes count (Figure 3) in the phytoremediated effluent exposed fish. This might also be due to decreased toxic stress of the decontaminated effluents following its phytoremediation by the macrophytes. However values of the most of the haematological parameters of phytoremediated effluent exposed fish still continued to remain below the levels of control wild fish. Since the availability of metals to living organisms is known to be governed by the physicochemical properties of the water (Roditi *et al*., 2000; Elahee and Bhagwant, 2007). Therefore, significant improvement in the physicochemical properties of the phytoremediated effluent could possibly explain their less toxic effect on health of the fish.

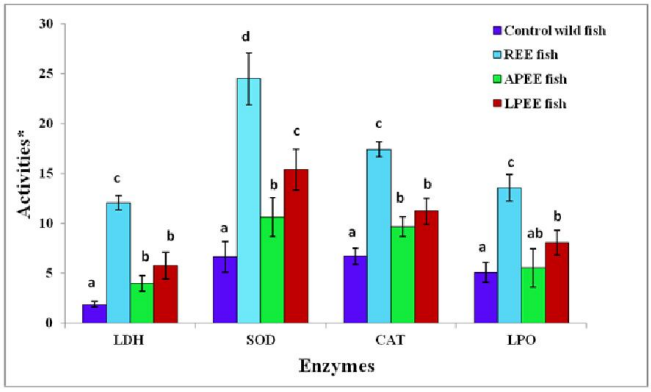

Figure 2. Enzyme activities in the fish exposed to phytoremediated effluent & its comparison to those of raw effluent exposed ones and wild controls

*denotes units of activities- LDH: IU/L; SOD: Unit/mL protein; CAT: n mole of H_2O_2 decomposed/min/mL protein; LPO: n mole of TBARS formed/min/mL protein.

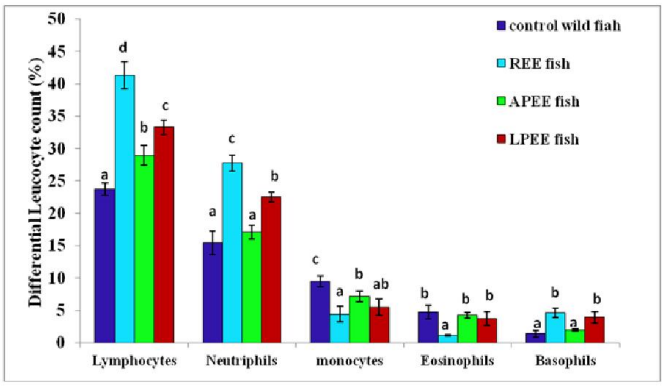

Figure 3. Alteration in the differential leukocyte count in the fish exposed to phytoremediated effluent & its comparison to those of raw effluent exposed ones and wild controls

Significant increase ($p<0.05$) in glucose and decrease in protein levels was noticed in the blood of fish exposed to raw effluent (Table 2). This was due to breakdown of proteins for generating additional amount of glucose to meet the enhanced energy demands for combating the toxicity stress of the effluent. Phytoremediation detoxified the effluent that caused substantially decrease in glucose levels of the fish (Table 2).

Table 1. Haematological parameters of the fish exposed to phytoremediated effluent

	RBC ($\times10^6$mm^{-3})	Hb (g/dl)	Ht (%)	WBC ($\times10^3$mm^{-3})	MCV (fl)	MCH (Pg)	MCHC (g/dl)	0_2 CC (ml (0^2/g^{-1}/Hb)
REE fish	1.14^a±0.04	4.433^a ±0.46	19.5^a ± 0.64	28.733^d ± 0.31	171.35^b ±8.78	38.873^b ± 3.78	22.71^a ± 2.14	5.542^a ± 0.58
APEE fish	2.48^c ±0.05	6.83^c ± 0.19	24.773^c ± 0.61	23.25^b ±0.25	99.953^a ± 4.44	27.56^a ± 1.34	27.57^b ± 0.81	8.537^c ± 0.23
LPEE fish	2.11^b ±0.04	5.57^b ±0.31	22.15^b ± 0.38	25.437^c ± 0.41	102.26^a ± 4.80	26.341^a ± 2.01	25.15^a ±1.54	6.962^b ± 0.39
Control	3.1^d ± 0.16	8.367^d ±0.69	30.67^d ± 0.34	19.233^a ± 0.42	99.28^a ± 6.80	27.047^a ± 1.70	27.28^b ± 1.6	10.46^d ± 0.69

Abbreviations: REE: Raw effluent exposed, APEE: *Azolla* phytoremediated effluent exposed, LPEE: *Lemna* phytoremediated effluent exposed

Table 2. Biochemical parameters of the fish blood exposed to the phytoremediated effluent

	Glucose (mg/dl)	Protein (mg/dl)	Cholesterol (mg/dl)	Total Lipid (mg/dl)
REE fish	110.08± 3.12^d	4.16± 0.81^a	73.447± 2.01^d	307.33 ± 4.72^a
APEE fish	90.497± 0.93^b	23.60± 1.99^c	60.857± 0.66^b	409.67 ± 4.04^c
LPEE fish	99.873± 1.3^c	17.76± 1.67^b	67.516± 2.02^c	371.82 ± 3.05^b
Control	73.187± 1.62^a	31.23± 1.79^d	49.04 ±1.04^a	426.79 ± 5.72^d

Abbreviations: REE: Raw effluent exposed, APEE: *Azolla* phytoremediated effluent exposed, LPEE: *Lemna* phytoremediated effluent exposed

Table 3. Enzyme activities in the fish blood exposed to the phytoremediated effluent

	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
REE fish	77.241± 1.48^d	79.165± 1.72^d	44.629± 2.35^d
APEE fish	52.957± 1.6^b	55.333 ±2.46^b	31.758 ±1.74^b
LPEE fish	58.532± 0.58^c	64.479±1.2^c	37.969 ± 1.16^c
Control	37.805 ± 1.24^a	43.650±1.96^a	23.025± 0.35^a

Abbreviations: REE: Raw effluent exposed, APEE: *Azolla* phytoremediated effluent exposed, LPEE: *Lemna* phytoremediated effluent exposed

The decrease was however below the levels of untreated control fish which might be due to persistence of contaminants in both the phytoremediated waste waters. The elevated levels of glucose provide extra energy to counter the toxicity stress of the incompletely decontaminated effluents. The levels of serum proteins in the fish exposed to both the phytoremediated effluents increased ($p<0.05$) when compared to that of raw effluent exposed ones (Table 2). This was due to decreased stress of the toxicity of the decontaminated effluent. The levels of total lipid and cholesterol increased in the serum following exposure to raw effluent (Table 2). The increased cholesterol and lipids level might have occurred due to their release from the cell membrane of damaged tissues of raw effluent exposed fish. Increased level of serum cholesterol has also been noticed in the blood of *Channa punctatus* (Kaur and Kaur, 2006) and *Cirrhina mrigala* (Kumar *et al*., 2005) due to different toxicants exposure.

In phytoremediated effluent exposed fish the cholesterol and lipid levels also improved (lowered) ($p<0.05$) (Table 2). Decreased levels of cholesterol and lipids indicate less severity of toxicity of both the phytoremediated effluents. Activities of different enzymes (AST, ALT and ALP) have extensively been used as important biomarkers to illustrate acute hepatic damages. Hence bioassay of these enzymes is often used as diagnostic tools to evaluate the liver activities. PMN effluent caused increased activities of AST, ALT and ALP in the serum of *L. rohita* indicating infliction of damage suffered by the hepatic tissue (Table 3). Increased activity of LDH also indicates compensatory increase in cellular metabolism and thereby more production of lactic acid causing acidosis (Figure 2). Following exposure to the phytoremediated effluents the activities of these enzymes were found to be lower than the levels obsereved in raw effluent exposed fish (Table 3). Decreased activities of these enzymes in phytoremediated effluent exposed fish points towards improvement in the health of the fish. However their levels continued to be above the levels of untreated control fish. Metals and other xenobiotics can 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 (Pinto *et al*., 2003; Gabriel *et al*., 2009). Due to their inhibitory effects, SOD-CAT system is known as the first line of defence against oxidative stress (Nanda *et al.*, 2010; Atli and Canli, 2010). Raw effluent exposure caused increased activities ($p<0.05$) of SOD and CAT in the serum (Figure 2). This might to protect the tissue damage caused due to high loads of metals and other xenobiotics accumulated in the tissues of raw effluent exposed fish. Enhanced activity of LPO in raw effluent exposed fish also indicates the ROS-induced peroxidation leading to the destruction of cell membranes (Figure 2). Activities of all these enzymes improved (lowered activity) ($p<0.05$) in fish exposed to both the phytoremediated effluents.

This is might be due to less absorption of metals by the tissues leading to lowered oxidative destruction. However activities of these enzymes could not reached to the levels of the wild fish perhaps due to incomplete decontamination of metals from the effluent by the macrophytes. In conclusion exposure of fish to detoxified effluents caused significant improvement in the different blood profiles in comparison to raw effluent exposed ones. However in most of the cases their levels did not reach to those of wild control fish. This indicates that following phytoremediation effluent became less toxic, but is still continued to remain unsuitable for fish culture. Partial improvement by phytoremediation analyzed by fish bioassay also revealed the shortcoming of phytoremediation technology. This drawback might help the botanists & toxicologist to further improve the technology of phytoremediation

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