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Toxicity Analysis of Effluent Released During Recovery of Metals from Polymetallic Sea Nodules Using Fish Haematological Parameters

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1. Introduction

To meet its domestic demands India has to import many of the economically important metals like Manganese, Iron, Cobalt, Zinc, Nickel and others. As the land based resources of these metals are depleting very fast, considerable efforts have been made to extract metals from pollymetallic sea nodules (PMN) during the past four decades all over the world. To attain self sufficiency scientists at National Metallurgical Laboratory, Council of Scientific and Industrial Research, Jamshedpur, India has developed an indigenous process to recover some of these metals from PMN. The PMN are rock concretions on the sea bottom formed of concentric layer of iron and manganese hydroxides around their core. They are small, slightly flattened, dark-brown coloured balls measuring 5 to 10 centimeters in diameters. The chemical composition of the nodules varies considerably according to the kind of minerals and the size and characteristics of the core. Those of the greatest economic interest contain Mn (27-30%), Ni (1.25-1.5%), Cu (1-1.4%), and Cobalt (0.2-0.25%). Other constituents include Fe (6%), Si (5%), Al (3%) with lesser amounts of Ca, Na, Mg, K, Ti, and Ba along with hydrogen and oxygen. For metal extraction purpose nodules are dried at 110^0 C, grounded and treated with reducing agents, followed by ammonia leaching. Separation of metals is done by the process of solvent extraction and electrowining (Jana *et al*., 1990; Kumar *et al*., 1990; Agarwal & Goodrich, 2008; Biswas *et al*., 2009). During the process of metal recovery highly contaminated effluent is generated that still retains substantial amount of metals (Vaseem & Banerjee, 2011a). These metals are highly toxic and are one of the main causes of environmental pollution. Two most important factors that contribute to the deleterious effects of heavy metals as pollutants are their non-degradation in the nature (unlike organic pollutants) and their tendency to bioconcentrate and settle at the bottom of water bodies. Hence our main aim has been to monitor the toxicity rendered to the aquatic ecosystem by this highly contaminated effluent (Table 1) using fish as an experimental model. *Labeo rohita* (commonly known as Rohu), a major Indian carp of great nutritional importance has been selected for the toxicity analyses of the effluent because fishes have widely been used as effective bioindicator. This graceful Indo-Gangetic riverine species is one of the three important major carps of the Indian subcontinent belonging to the family cyprinidae. It is the natural inhabitant of the wetlands of northern and central India, and the rivers of Pakistan, Bangladesh and Myanmar. It is a diurnal, herbivore and generally

solitary species. It attains sexual maturity within two years. In nature it spawns in the marginal areas of flooded rivers. Due to its wider feeding niche, rohu is usually stocked at relatively greater quantity than the other two carps *Catla catla* and *Cirrhina mrigala*. Higher consumer preference and market demand for rohu during recent years have also led to the increased practices of culture of this fish species. There has always been a chance of metal toxicity of this fish through contamination of the water bodies by various polluting agents generated through anthropogenic activities.

Heavy metals	**Conc. (mg/l) In raw effluent**	**Standard (EPA) 2003 (mg/ml)**
Mn	4.957 ± 0.130	0.2
Cu	1.432 ± 0.013	0.5
Zn	0.816 ± 0.013	2.0
Fe	0.762 ± 0.02	2.0
Pb	0.655 ± 0.017	0.05
Cr	0.07 ± 0.001	0.05
Cd	0.018 ±0.0004	0.01
BOD	182 ± 3	40
pH	5.2 ± 0.5	5-9
Sodium	130.26 ± 1.96	200
Potassium	3.42 ± 0.07	n.a
Sulphate	2300 ± 4.515	750
DO (mg/l)	18.8 ±0.821	n.a
CO_2	28.60 ± 0.418	n.a
Carbonate	296 ±2.2	n.a

Table 1. Physicochemical properties of sea nodule effluent.
Source: Vaseem and Banerjee (2011 a).
Abbreviations. n.a: data not available; Values are given in mean ± SD

Amongst the various tissue components employed for toxicity estimation, the blood parameters of the fish have been selected for the study because ambient contaminants often produce rapid changes in the blood characteristics (Carvalho & Fernandes, 2006). Several haematological indices haematocrit (Ht), haemoglobin (Hb), total erythrocyte count (TEC), total leukocyte count (TLC) have successfully been applied in the past to assess the functional status and oxygen carrying capacity of blood stream of variously exposed fishes (Shah & Altindag, 2004). Kori *et al* (1991) observed decreased Hb, Ht, RBC counts in copper exposed *Clarias isheriensis*. Das and Mukhrjee (2000) observed decreased Hb, TEC and serum protein content and increased TLC and blood glucose concentration of the quinolphos exposed carp, *L. rohita*. Decreased Hb, TEC, packed cell volume (PCV) and mean corpuscular haemoglobin concentration (MCHC) and increased white blood cell count and MCV have been noticed in *L. rohita* collected from the polluted lakes of Bangalore, Karnataka India (Zhushi *et al.*,2009). Kavitha *et al* (2010) observed decrease in various haematological parameters (Hb, Ht, RBC, WBC, plasma glucose, plasma protein) in arsenate treated Indian major carp, *Catla catla*. They however observed increased corpuscular indeces like MCV, MCH and MCHC in the same fish. Hence in the present study analyses of these blood parameters of *L. rohita* have been applied to evaluate the toxicity of the sea nodule effluent.

2. Materials and methods

2.1 Experimental design

Healthy specimens of the freshwater teleost *Labeo rohita,* belonging to the family Cyprinidae, were collected from the hatchery at Banaras Hindu University, Varanasi, India. The fish were acclimatised to the laboratory conditions (temperature 26.0^0 C ± 2.0) for one month in tap water (pH: 7.2 ± 0.3; salinity: 0.3 ± 0.08 pg/l, dissolved oxygen: 6.6 ± 0.06 mg/l, total alkalinity: 21.0 ± 8.0 mg/l, and total hardness: 16.0 ± 0.04 mg/l) in 40 litre plastic tubs. The fish were fed *ad libitum* with rice bran and groundnut oil cake (2:1) twice daily. Water in the tubs was aerated and changed after every 24 hrs. Batches of 15 fish (weight of 28–30 g and 11–12 cm in length) were exposed to the 20 litre of the raw effluent. Parallel batches of control fish (15 in each batch) were exposed to plain tap water under identical laboratory conditions. Ten randomly selected fish each from experimental as well as control aquaria were sacrificed after 10 and 20 days of exposure for haematological as well as biochemical analyses.

2.2 Collection of blood

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.

2.3 Haematological analysis

Blood samples from the control as well as experimental groups of fish were subjected to determination of Hb, Ht, RBC and WBC count. Haemoglobin was estimated by using the Sahli's hemoglobinometer. Oxygen carrying capacity of the fish blood was calculated by multiplying the haemoglobin content by 1.25 oxygen combining power of Hb/g (Johansen, 1970). Erythrocyte and leukocyte counts were studied by Neubauer's improved hemocytometer using Hayem's and Tuerk's solution as a diluting fluid, respectively (Samuel, 1986). Hematocrit values were measured by Wintrobe's methods. Mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and mean corpuscular volume were calculated by the standard formulae suggested by Dacie and Lewis (1991). The remainder of the blood was centrifuged for 15 min for biochemical analysis of the serum.

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

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

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

2.4 Biochemical analysis

The concentrations of serum glucose, cholesterol and protein were estimated by the methods of Seifter *et al*. (1950), Zlatkis *et al*. (1953), and Lowry *et al.* (1951) respectively.

2.5 Statistical analysis

For statistical analyses one-way analysis of variance (ANOVA) was performed to determine significance of differences ($p < 0.05$) between the pairs of means. Duncan's multiple range test (DMRT) was also applied ($p < 0.05$) to find out which means are significantly different from others. Since there were no significant variations between the values obtained from various control groups, the average value of all the control groups was taken into account. In table 2, 3 & 4 alphabets denote results of DMRT. Different alphabets show significant changes in various parameters in control and exposed fish ($p < 0.05$).

3. Results

The Hb content (g/dl) of the unexposed control fish (*L. rohita*) was 8.367 ±0 .694. Following exposure it started decreasing and was 6.2± 0.216 after 10 days and 4.43 ± 0.464 after 20 days. Both the decreases were statistically significant ($p<0.05$) (Table 2).

The haematcrit value (%) of control fish was 30.66±0.339. In exposed fish it decreased significantly ($p<0.05$) becoming 0.26± 0.163 after 10 day and 19.5± 0.637 after 20 days (table 2).

Following exposure the total erythrocyte count (10^6 mm^{-1}) decreased significantly ($p<0.05$) and became 2.367 ± 0.205 from 3.1± 0.163 (control) after 10 days. It continued to decrease further becoming 1.14 ± 0.043 after 20 days (Table 2).

The total leukocyte count increased significantly ($p<0.05$) following exposure (Table 2). After 10 days of exposure TLC of the fish became 25.32 ± 0.891 (from 19.23 ± 0.418 in control). It increased further and became 28.73 ±0.309 after 20 days.

The MCV, MCH, MCHC and O_2 carrying capacity of blood deduced from the above mentioned haematological data has been detailed in table 3.

Pattern of changes in the serum glucose, cholesterol and protein levels have been shown in table 4. The serum glucose content (mg/dl) increased significantly ($p<0.05$) from 73.19 ± 1.617 in control to 77.733 ± 0.329 after 10 days and 93.006 ± 2.163 after 20 days of exposure.

The serum cholesterol level (mg/dl) also increased from 49.04 ± 1.043 in control to 56.52 ± 1.79 and 73.446 ± 2.011 after 10 and 20 days of exposure respectively.

Total serum protein level (mg/dl) in control fish was 3.266± 0.169. While it decreased to 2.9 ± 0.081 on 10 days of exposure. It became 1.166 ± 0.124 after 20 days. The decrease in protein concentration was statistically significant ($p < 0.05$) for both the values: after 10 and 20 days of exposure.

Parameters	Control	10 days exposed	20 days exposed
Total erythrocyte count (×106 mm^3)	3.1 ± 0.163^a	2.367 ± 0.205^b	1.14 ± 0.043^c
Total leukocyte count (×103 mm^3)	19.233 ± 0.418^a	25.32 ± 0.081^b	28.73 ± 0.309^c
Haemoglobin (g/dl)	8.367 ± 0.694^a	6.2 ± 0.216^b	4.43 ± 0.464^c
Haematocrit (%)	30.66 ± 0.339^a	26 ± 0.163^b	19.5 ± 0.637^c

Values are given in mean ± SD

Table 2. Alteration in haematological parameters in sea nodule exposed *L. rohita.*

Parameters	Control	10 days exposed	20 days exposed
Glucose (mg/dl)	73.187 ± 1.617[a]	77.73 ± 0.329[b]	110.08 ± 3.16[c]
Cholesterol (mg/dl)	49.04 ± 1.043[a]	56.52 ± 1.795[b]	73.446 ± 2.011[c]
Protein (mg/dl)	31.23 ± 1.827[a]	29 ± 2.32[a]	4.16 ± 0.812[b]

Table 3. Alteration in Biochemical parameters of blood in sea nodule exposed *L. rohita*. Values are given in mean ± SD

Parameters	Control	10 days exposed	20 days exposed
MCV (fl)	99.276 ± 6.809[a]	110.73 ± 10.09[b]	171.356 ± 8.77[c]
MCH (pg)	27.047 ± 1.703[a]	26.313 ± 2.16[a]	38.873 ± 3.779[b]
MCHC (g/dl)	27.28 ± 1.60[a]	23.853 ± 1.89[a]	22.71 ± 2.145[a]
O2 carrying capacity Of blood (ml O_2/g^{-1}/Hb)	10.458 ± 0.690[a]	7.75 ± 0.568[b]	5.54 ± 0.580[c]

Table 4. Alteration in calculated haematological indices in sea nodule exposed *L. rohita*. Values are given in mean ± SD

4. Discussion

Blood parameters are considered as a patho-physiological indicator of the entire body and therefore are important in diagnosing the structural and functional status of fishes exposed to toxicants (Adhikari & Sarkar, 2004; Maheswaran et al., 2008).

Estimation of haemoglobin was employed because this blood component is a part of the sophisticated oxygen delivery system that provides the desired amount of oxygen to the tissues under a wide variety of circumstances (Voet & Voet, 1990). The oxygen transport function of blood is the product of a complex integration of the effects of various physicochemical factors such as temperature and the concentrations of allosteric co-factors, dissolved gases, protons and other ions on the oxygen binding properties of haemoglobin (Weber & Lykkeboe, 1978; Weber, 1982). According to Blaxhall and Daisley (1973) the determination of haemoglobin concentration can be a good indicator of anaemic conditions in fish. A review of table 2 suggests that the PMN effluent causes loss of Hb leading to anaemic condition to the fish after 20 day of exposure. Cyriac et al. (1989) considered decreases in haemoglobin concentration as a contribution to haemodilution. Haemodilution is a mechanism that reduces the concentration of the pollutants in the circulatory system (Smit et al. 1979). Similar haemodilution has also been observed in fish contaminated with aluminium, copper, manganese and zinc (Torres et al., 1986; Wepener, 1990; Nussey, 1994; Coetzee, 1996; Barnhoorn, 1996). The decrease in haemoglobin concentration signifies that the fish's ability to provide sufficient oxygen to the tissues is restricted considerably and results in decreased physical activity (Grobler, 1988; Wepener, 1990; Nussey, 1994). According to Reddy and Bashanihideen (1989) this significant decrease in the haemoglobin concentrations of fishes under toxic stress might be due to either an increase in the rate at which the haemoglobin is destroyed or due to decreased rate of haemoglobin synthesis. Other reason for the progressive reduction in the haemoglobin content might be attributed to depression/exhaustion of haemopoietic potential of the fish (Sawhney & Johal, 2000).

Suppression of haemopoietic activity of the kidney in addition to the increased removal of dysfunctional red blood cells might be the third reason for the decreased Hb content (Stormer et al., 1996). Devi and Banerjee (2007 a, b) also noticed anaemic condition of ammonia and lead exposed *Channa striata* due to deceased levels of Hb, TEC, TLC, Ht and cellular degeneration of RBCs.

Haematocrit (measurement of packed erythrocytes) is an important instrument for determining the amount of plasma and corpuscles in the blood and used to determine the oxygen carrying capacity of the blood (Larsson et al., 1985). It is also defined as the volume occupied by erythrocytes in a given volume of blood. In fish the haematocrit reading is valuable in determining the effect of stressors on their health. (Munkittrick & Leatherland, 1983). Significant decreases in the haematocrit values (Table 2) following exposure to the sea nodule effluent also suggests anaemia and haemodilution possibly due to gill damage or/and impaired osmoregulation (Larsson et al., 1985).

Erythrocytes are produced in the haematopoietic tissue, which is situated in the spleen and head kidney (Smith, 1982; Grey & Meyer, 1988; Kita & Itazawa, 1989; Heath, 1995). It is well known that a reduced quantity and quality of erythrocytes and a decreased haemoglobin level as also noticed in the present study could lead to deleterious oxygen transport. Extensive reduction in haemoglobin content due to any blood dyscrasia and degeneration of the red blood cells could be ascribed as pathological conditions in fishes exposed to toxicants leading to deteriorated oxygen supply (Buckley et al., 1976). Decrease in TEC in the present study (Table 2) might be due to inhibition of RBC production or Hb synthesis. Exposure to other toxicants also causes decrease in erythrocyte counts in fishes (Van der Merwe, 1992, Omoregie *et al*., 1990; Das & Mukherjee, 2000).

The calculated haematological indices, MCHC, MCH, and MCV are other important indicators in the diagnosis of anaemia in most animals (Coles, 1986). Alterations in these haematological parameters (increase MCV and MCH, decrease of MCHC) might be due to a defence against the toxic effect of the effluent through the stimulation of erythropioesis or due to the decrease in RBCs, Hb and Hct values following disturbances in both metabolic and haemopoietic activities of the fish exposed to different concentrations of pollutants (Abd-Alla *et al.,* 1991; Mousa, 1994). The increase in MCV value might be due to increased number of immature RBC (Carvalho & Farnandes, 2006). Increase in MCV and MCH along with slightly diminished MCHC values (Table 3) suggest the macrocytic nesmochromic type of anaemia. Similar toxicological response is also recorded in common carp caused by acute effect of phenitrothion, imidan, and dichlorvos (Svobodova., 1971) and Svobodova and diazinon (Svoboda et al., 2001). Changes in haematological parameters of *C. gariepinus* due to stress caused by environmental pollutants, diseases or attack by pathogens have also been reported by a number of workers (Ezeri, 2001, Gabriel et al., 2001).

White blood cells (WBC), or leukocytes, are important component of the immune system involved in defending the body against both infectious diseases and foreign materials. Five different and diverse types of leukocytes exist, but they are all produced and derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell. Leukocytes are found throughout the body, including the blood and lymphatic system. The increased total leucocytes count of the exposed fish in the present study indicates increased defensive reaction against the stressors. Increased WBC count have also been reported in

fishes exposed to other certain xenobiotics like endosulfan (Abidi & Srivastava,1988), aflatoxin B (Lovell & Jantrarotai,1991), crude oil (Khadre & Shabana,1991) and to industrial effluents (Wahbi, 1992).

While analysing different haematological parameters in the freshwater fish, *Heteroclarias* sp. (Osteichthyes: Clariidae) exposed to sublethal concentration of zinc, Kori-siakpere *et al* (2008) noticed decreased values of Hb, Ht, RBC, WBC, protein and glucose and calculated hametogical parametres. Gabrial et al (2007) observed haematological changes in the catfish *Clarias gariepinus* following 14-days of exposure to refined petroleum oil, kerosene. The results include decreased values of Hb, Ht, WBC and MCV and increased levels of MCHC, MCH, neutrophils, monocytes and thrombocytes.

The presence of toxicants in aquatic ecosystem also exerts its effect at cellular or molecular levels which results in significant changes in biochemical compositions of the organisms. Due to metal complex formation, normal functioning of cells are disturbed that in turn results in disturbed physiological and biochemical equilibrium of animals (Gagnon et al, 2006; Vaseem & Banerjee, 2011b). The influence of the stressors on carbohydrate metabolism of fish includes alterations in glucose, glycogen and lactic acids contents. Among these parameters the analyses of blood glucose level (Table 4) have been used as an effective indicator to monitor the stress condition of the animal including fish. The elevated glucose level in the blood stream in this study might be due to gluconeogenesis to supplement additional energy needed to meet the increased metabolic demands (Zutshi *et al*., 2009; Kavitha *et al*., 2010). While estimating the carbohydrate, lipid and protein concentrations in six vital organ system of *Labeo rohita* exposed to sea nodule effluent, Vaseem and Banerjee (2011b) noticed depletion of these macromolecules in all the six tissues after 20 days of exposure. They postulated that the decrease might be due to mobilization of these macromolecules to maintain steady supply of energy in the serum to negotiate the stress during the entire period of exposure. This could also be the reason for increased level of glucose in the serum of sea nodule effluent exposed *Labeo rohita* (Table 4).

The reduction in serum protein levels in sea nodule effluent (table 4) exposed *L. rohita* might be due to breakdown of proteins and other macromolecules for several known (e.g. to meet the higher energy demand during the prevailing stress (Zutshi *et al*., 2009) or might be due to liver cirrhosis or nephrosis or due to alteration in enzymatic activity involved in protein biosynthesis as suggested by Nandi *et al*., 2005, Yousef *et al*., 2008, and Palaniappan & Vijjayasundarum. 2009) or unknown reasons.

The blood cholesterol level in the present study increased significantly after both (10 as well as 20) days of exposure (Table 4). Increased cholesterol level might have occurred due to dysfunction of liver causing release of additional quantities of cholesterol into the blood. Increased level of cholesterol has also been reported in the serum of *Channa punctatus* (Kaur & Kaur 2006) and *Cirrhina mrigala* (Kumar *et al.,* 2005) due to exposure to other toxicants.

5. Conclusion

The results of the present investigation indicate that exposure to sea nodule effluent induces significant changes in the haematological and biochemical profile of the Indian major carp *L. rohita*. Prolonged exposure of sea nodule effluent also affected the survival of fish. Our data

illustrated the toxicological impact of effluents having a variety of contaminants especially the toxic metals in different concentrations.

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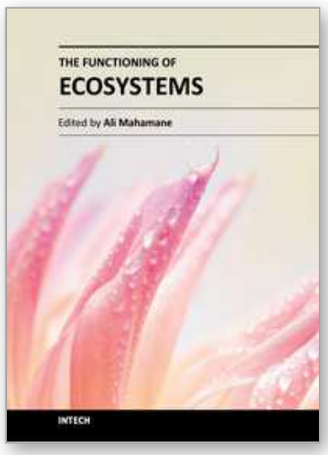

The Functioning of Ecosystems

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The ecosystems present a great diversity worldwide and use various functionalities according to ecologic regions. In this new context of variability and climatic changes, these ecosystems undergo notable modifications amplified by domestic uses of which it was subjected to. Indeed the ecosystems render diverse services to humanity from their composition and structure but the tolerable levels are unknown. The preservation of these ecosystemic services needs a clear understanding of their complexity. The role of the research is not only to characterise the ecosystems but also to clearly define the tolerable usage levels. Their characterisation proves to be important not only for the local populations that use it but also for the conservation of biodiversity. Hence, the measurement, management and protection of ecosystems need innovative and diverse methods. For all these reasons, the aim of this book is to bring out a general view on the biogeochemical cycles, the ecological imprints, the mathematical models and theories applicable to many situations.

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