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Bioaccumulation of Heavy Metals and Physiological Response in *Anabas testudineus* on Exposure to Paper Mill Effluent

Prasanta Nanda*

Department of Zoology, D. N. Government College, Itanagar, Arunachal Pradesh 791113, India

Abstract

Bioaccumulation of heavy metals and biochemical response in *Anabas testudineus* was investigated after exposing it to various concentrations (0 - 40%) of paper mill effluent containing low level toxic metal ions. Maximum amount of zinc was accumulated in gills, however ovary was found to be the main target organ for Zn accumulation based on maximum accumulation factor. The increased content of metal ion in all the tissues at higher exposure level confirms the dose dependent accumulation of metal ion. Zinc accumulation follows the pattern gill> liver> ovary> muscle. Copper accumulation was highest in liver and the order of accumulation follows the trend gill> liver> muscle> ovary. Cadmium bioaccumulation was significantly higher in all the tissues without showing any specific trend. Maximum cadmium and lead accumulation was observed in ovary. The trend of Pb accumulation followed the order ovary> liver> gill> muscle. In terms of the type of metal accumulation in all the tissues together, the trend was Zn> Pb> Cu> Cd while tissue specific total metal accumulation followed gills> liver> ovary> muscle. The result of this study indicates that ovary acts as a major accumulator of heavy metals besides gill and liver in *A. testudienus* when exposed to paper mill effluent. Hematological parameters viz., Hb, TEC, hematocrit and MCHC showed significant ($p<0.05$) decrease in the exposed fish in comparison to the control value. While level of glucose (except liver) and protein depleted consistently, cholesterol level showed significant increase in plasma, liver and muscles. Physiological response upon exposure to PME indicates damage of tissues with loss of protein and impaired regulation of metabolic function as indicated by blood parameters.

Keywords: Bioaccumulation; Heavy metals; Paper mill effluent; Maximum accumulation factor; Physiological response; *Anabas testudineus*

Introduction

During last few decades, there has been considerable concern over heavy metals contamination of aquatic environment and the potential health threat to public potable water sources. The non-degradable and persistent nature of the metal ions results in longer exposure and accumulation of these substances in the aquatic flora and fauna. This would result in deterioration and disturbance of aquatic ecosystem. Heavy metal accumulation in the aquatic environment could result in toxicity to both aquatic life and human. Edible fish present in aquatic bodies form an important group of organism as heavy metal once accumulated in fish tissues could act as a potential carrier of metal ion along the food chain. At the end, directly or indirectly the metal ion in the aquatic medium reaches to the man. Hence several studies involving bioaccumulation of heavy metals have been conducted in fishes [1-4] found in river streams generally receiving industrial effluents containing toxic heavy metals and organic pollutants.

Accumulation of metals (Cu, Zn, Cd, Ni, Cr, Pb) in different tissues viz., blood, gill, gut, liver, muscle, kidney, ovary and gonad etc., have been extensively investigated in various fishes [3,5-9]. Most of these studies report metal accumulation indicating preference of the tissues for some metals over the others. The characterization of the accumulation of metals into different organs has proven to be a representative measure of the heavy metal exposure [10] and is used to monitor the bioavailability of these metals. Gills and livers are considered most important for assessing metal accumulation. Since gill is in direct contact with metal present in the water, the concentrations of metals in gills reflect the concentration of metals in water. Copper accumulation was found to be maximum in gills in *O.niloticus* [11] whereas highest Cd accumulation in gill was observed during long term sub lethal Cd exposure in rainbow trout [5]. The metal accumulation in liver and its concentration represent storage of metals contributing substantially to the overall metal load in the body. In addition to gill and liver, ovary involved in reproduction of fish is a metabolically active tissue and accumulate heavy metals of higher levels [7]. Amongst all the tissues in fish, muscle is not known as an active metal accumulator. Besides accumulation in tissues, changes in hematological and biochemical parameters have also been reported in several such studies [2,11-15]. These investigations have helped to understand the diversity in mechanism of heavy metal homeostasis in fishes although no universal mechanism could be established.

We conducted a series of study involving exposure of paper mill effluent (PME) containing trace level of heavy metals on *Anabas testudineus*, the climbing perch. *Anabas testudineus* (family, Anabantidae), was selected for this study since it is a popular edible fish widely available in the upper stream of river Ib, located at Brajarajnagar, in Jharsuguda district of Orissa state (India) which receives industrial discharge from the nearby situated paper mill effluent. Chemical analysis of paper mill effluent indicated presence of different heavy metals viz., Cd, Pb, Cu, Zn etc., at a concentration less than 0.2 mg/L [16]. Our result on toxicity study showed that *A. testidineus* was more sensitive to the exposure of the paper mill effluent in comparison to the other two air breathing fishes viz. *Clarias batrachus* and *Channa punctatus* as indicated by their LC_{50} value [17]. Further investigation

***Corresponding author:** Prasanta Nanda, Department of Zoology, D. N. Government College, Itanagar, Arunachal Pradesh 791113, India, Tel: +91-0360-2291809; E-mail: nandap71@rediffmail.com

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in the same species showed substantial histopathological abnormalities including damage of hepatocytes, gills and intestinal villi upon exposure to the paper mill effluent [18]. In view of this, there is a need to investigate the cause of such damage to various organs when the fish is exposed to paper mill effluent. One of the possibilities could be presence of toxic metal ions. Though lesser in concentration, the toxic metal ions can damage the various tissues and organs if it is bio-accumulated. Therefore in the present study, investigation were carried out to find out the level of accumulation of different metal ions in these tissues and overall physiological (hematological and biochemical) response as an important aspect of understanding the toxic effect of paper mill effluent.

Materials and Methods

Experimental set-up

The paper mill effluent was collected from the integrated pulp and paper mill situated near the river Ib (Jharsuguda, Odisha, India). The concentration of different heavy metals found in the paper mill effluent was ≤ 0.2 ppm [16]. The paper mill effluent was diluted with tap water to obtain different concentrations (5, 10, 20, and 40%) for further use. The tap water was used as a control (0% effluent) that did not contain any detectable heavy metals included in this study. Toxicity of the paper mill effluent (LC_{50}) on different air breathing fish has shown *Anabas testudineus* with highest tolerance [17]. Therefore, this fish was selected to carry out the current study. Healthy specimens of *A. testudineus* were collected from the upper stream, i.e., above the point at which the paper mill effluent enters into the river Ib (Odisha, India). The weight and length of the specimens selected for the study was in the range of 20-22 g and 10-12 cm respectively. Each set of treatment included exposure of 10 adult specimens in a glass aquarium (50 cm × 50 cm×45 cm) containing 20 L of paper mill effluent of desired concentration for 30 days. Temperature was maintained at 26 ± 2°C, aeration was continuous and medium static. The medium was replaced every 3 days over the 30 days period and fish were fed with commercial fish food.

Tissue metal analysis

At the end of the exposure period, gill, liver, muscle, and ovary were dissected out. The tissues were oven dried at 60°C till constant weight. After determination of the dry weight the tissues were digested at 70°C in an aluminium block heater in screw capped polypropylene tubes with a mixture of 2 ml HNO_3 and distilled water (1:1 V/V). The heavy metal (Cd, Cu, Pb, and Zn) contents of the tissue digests were determined using an Atomic Absorption Spectrophotometer (GBC-902). Atomic absorption standards procured from Sigma were used in the analysis. The amount of metal accumulation was expressed as µg metal ion/g dry weight of the tissue. Based on the metal accumulation value, the term 'maximum accumulation factor' was derived. This represents ratio of highest metal accumulation in tissues of the effluent exposed fish and accumulation in the same tissues of the control fish.

Hematology and tissue biochemistry

At the end of exposure period blood (1.5-2.0 mL) was collected from the caudal peduncle artery into heparinised tubes. Hemoglobin (Hb), total erythrocyte count (TEC), hematocrit and total leucocyte count (TLC) were determined and mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and mean cell volume (MCV) were calculated following the standard protocol (Dacie and Lewis). Plasma was separated from cells by centrifugation of whole blood at 1500 x g for 10 min (4°C). Liver and muscle tissues were dissected out. Biochemical parameters viz., protein, glucose and cholesterol were determined spectrophometrically in blood plasma, liver and muscle tissues following the procedure of Lowry et al. [19], Trevelyan and Harrison [20] and Libberman-Burchard [21] respectively.

Statistical Analysis

All results expressed are mean ± standard deviation (n=10), i.e., each data points are average of 10 separate fish samples. The differences in mean metal accumulation were analyzed using one-way analysis of variance (ANOVA). Statistical significance was assessed at $p<0.05$. All statistical operations were performed using Micrococal Origin (Version, 6.0).

Results

In our previous study, three types of air breathing fish found in the river viz., *Clarius batrachus*, *Chana punctatus* and *Anabas testudienius* were evaluated for their tolerance. When all these fish were exposed to Paper Mill Effluent, A. *testudineus* showed maximum LC_{50} [17] and therefore being most tolerant the same fish was used in the present study.

Metal accumulation in tissues

Accumulation of zinc (µg/g dry weight) in different tissues is presented in Figure 1. The highest quantity of zinc (332.9 ± 2.9, µg/g dry weight) was accumulated in gill exposed to 40% paper mill effluent and this was significantly (P<0.05) higher from other tissues (liver, ovary, and muscles). High level of Zn accumulation (µg/g dry weight) was also found in liver (283.4 ± 9.4) and ovary (231.8 ± 7.0). Muscle accumulated lowest quantity of zinc (56 ± 0.6 µg/g dry weight) among all the tissues investigated. Zinc content in all the tissues increased with increasing concentration of the paper mill effluent and was highest in fish exposed to 40% effluent. The accumulation of zinc in gill, liver, and ovary tissues was significantly (P<0.05) different at all level of exposure (5-40%) in comparison to the control. The increased content of metal ion in all the tissues at higher exposure level has further confirmed the dose dependent accumulation of metal ion. Although the pattern of metal accumulation in the 40% effluent treated fish was gill> liver> ovary> muscle, the trend of maximum accumulation factor was different. The highest accumulation factor was observed in ovary (4.08) followed by gill (2.85), liver (2.53) and muscle (1.83). This result

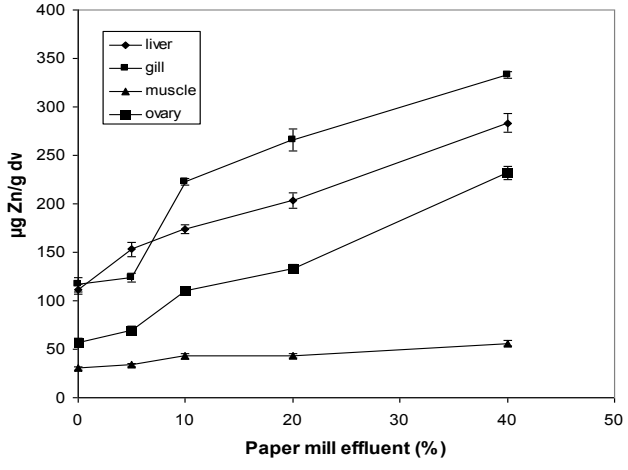

Figure 1: Zinc concentrations (µg/g dry weight) in tissues of the climbing perch after exposure for 30 days to paper mill effluent.

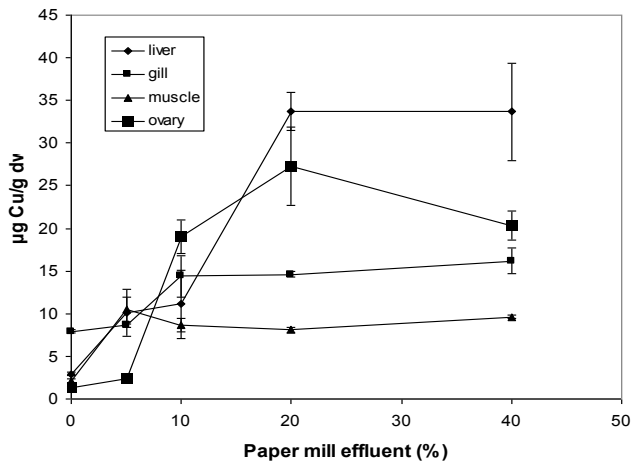

Figure 2: Copper concentrations (µg/g dry weight) in tissues of the climbing perch after exposure for 30 days to paper mill effluent.

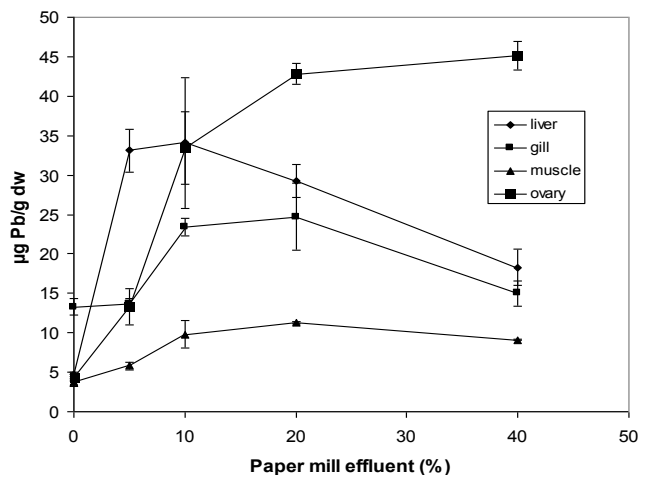

Figure 3: Cadmium concentrations (µg/g dry weight) in tissues of the climbing perch after exposure for 30 days to paper mill effluent.

indicate ovary as a target organ in terms of the number of times Zn was accumulated in effluent exposed fish in comparison to the control.

Copper accumulation was highest in liver (33.7 ± 5.7, µg / g dry weight) followed by ovary, gill and muscle (Figure 2). The distribution of copper in control group was gill> liver> muscle> ovary. This trend was completely different to the trend of accumulation, liver> ovary> gill> muscle observed in fish exposed to 40% effluent. Copper accumulation was significantly ($P<0.05$) high in all tissues and at all exposed concentration of effluent. However there was no significant difference between Cu accumulation in the gills of the fish exposed to 10 and 20% effluent. In muscle, the maximum amount of metal ion (10.5 ± 1.5, µg/g dry weight) was found in fish exposed 5% effluent. The accumulation of copper remained almost independent of concentration after this level of exposure. The maximum accumulation factor followed the trend ovary (15.11)> liver (11.79)> muscle (4.93)> gill (2.05).

Accumulation of cadmium in different tissues indicated the trend ovary> liver> gill> muscle (Figure 3). Maximum quantity of cadmium (7.3 ± 0.3 µg/g dry weight) was observed in ovary. The accumulation was only 1.5 ± 0.2 µg Cd/g dry weight in muscle. Cadmium concentration in the muscle tissues of the fish exposed to 5%, 10% and 20% effluent showed no significant ($P>0.05$) difference between each other, however they were significantly ($P<0.05$) different when compared to 40% effluent treated fish and the control. Similar trend was observed for cadmium accumulation in ovary of the fish exposed to 5%, 10% and 20% effluent. Interestingly, a sharp increase (43.75 %) in Cd accumulation was observed when the exposed effluent concentration increased from 20% to 40% in case of ovary tissue. Liver showed maximum accumulation (2.9 ± 0.8 µg / g dry weight) at 5% exposure, while maximum accumulation (2.3 µg/g dry weight) in gill was observed at 10% exposure level. The maximum accumulation factor followed the order muscle (5.84)> ovary (5.52)> gill (3.6)> liver (3.25). Though significantly higher accumulation was observed in these tissues at all exposed concentration no specific trend was observed.

The trend of Pb accumulation followed the order ovary> liver> gill> muscle (Figure 4). However, the maximum accumulation factor indicated the trend ovary (10.51)> liver (7.22)> muscle (3.07)> gill (1.86). Maximum accumulation of lead observed in ovary was 45.1 ± 1.8 µg / g dry weight. Metal ion content increased in ovary with increasing exposure concentration while in other tissues no particular trend was observed. In control tissues the accumulation of Pb followed gill> liver> ovary> muscle. Fish exposed to 5% effluent exhibited 7 fold enhancements in liver Pb concentration in comparison to the control. This level of Pb concentration built up was maintained in the liver in subsequent higher doses of exposure (10 and 20% effluent), which finally declined to 3.9 fold increase at 20% level. Similar trend of uptake was also observed in gills and muscles. Lead accumulation was significantly ($P<0.05$) different in comparison to the control in all the tissues at all exposed concentration except in gill at 5% exposure.

Hematology

Hematological parameters viz., Hb, TEC, hematocrit and MCHC showed significant ($p<0.05$) decrease in the exposed fish in comparison to the control value (Table 1). The changes in the hematological parameters were concentration dependent and least values were observed at highest exposure level (40%). While hemoglobin concentration decreased by 2.9 fold at 40% exposure level, the total erythrocyte count decreased by 3.7 fold that is reflected in enhancement of MCH value. In contrast, MCV increased by 1.6 fold at highest concentration of exposure (40% effluent). Total leucocytes

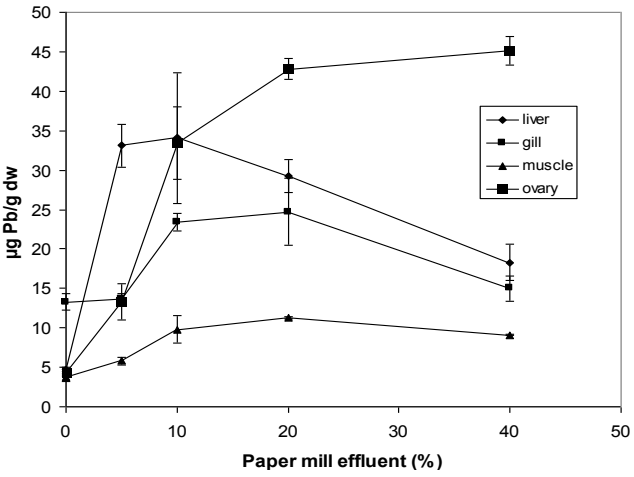

Figure 4: Lead concentrations (µg/g dry weight) in tissues of the climbing perch after exposure for 30 days to paper mill effluent.

Parameters	Paper mill effluent (%)				
	0	5	10	20	40
Hb (g%)	12.59 ± 2.4	11.67 ± 3.3	6.4 ± 2.7	4.7 ± 2.4	4.31 ± 0 .21
TEC (×10^6/mm^3)	2.6 ± 0.23	2.31 ± 1.4	1.78 ± 0. 2	0.78 ± 0.7	0.7 ± 0.4
Hematocrit (%)	45.43 ± 0.6	43.82 ± 0.13	33.75 ± 2.43	24.82 ± 0.13	19.53 ± 0.48
MCH(pg)	48.52 ± 3.02	50.42 ± 3.78	56.33 ± 3.99	60.14 ± 3.68	61.66 ± 5.88
MCHC (%)	27.74 ± 2.28	26.64 ± 1.5	19.2 ± 1.8	19.0 ± 1.71	21.16 ± 3.72
MCV (μ^3)	176.74 ± 21.9	189.35 ± 14.43	189.66 ± 32.04	316.87 ± 28.2	279.16 ± 54.03
TLC (× 10^3)	15.71 ± 2.43	16.39 ± 1.02	35.93 ± 2.25	44.06 ± 1.77	53.01 ± 2.97

Table1: Hematological parameter of *A. testudineus* exposed to paper mill effluent.

count increased with increasing effluent exposure concentration, finally gaining a 3.7 fold increase in cell numbers at 40 % exposure level.

Tissue biochemistry

Plasma, liver and muscle tissues were investigated for assessing the effect of metal accumulation on biochemical parameters such as glucose, protein and cholesterol. While level of glucose (except liver) and protein depleted consistently, cholesterol level increased significantly ($p<0.05$) upon increasing exposure to higher concentration of PME (5 - 40% concentration) (Figure 5a-c). There was sudden decline in the glucose content of PME exposed fish (40.3 ± 3.2 mg/dl) at lowest concentration (5%) from the control value (48.2 ± 4.9 mg/dl) in plasma. With further increase in concentration to 10% effluent glucose level decreased to 38.1 ± 3.5 mg/dl beyond which it remained constant at 32 mg/dl. Similar decreasing trend was observed for glucose level in the muscle. In contrast, glucose level increased with increasing effluent concentration with a maximum of 2.3 fold at highest concentration of exposure. Deproteination is clearly observed in fish exposed to PME of different concentration at all concentration across all tissues. The loss of protein was highest in muscle followed by plasma and liver. The effect was such that at higher concentrations (40%) only one fourth amount of protein remained in muscle. However cholesterol level shows a different trend. The overall increase in plasma cholesterol level was concentration dependant. There was a significant ($p<0.05$) increase in the plasma cholesterol level in PME (40%) exposed fish (423.9 ± 21.5 mg/dl) in comparison to the control fish (226.4 ± 10.5 mg/dl). Similarly increasing trend in cholesterol level was observed for liver and muscle.

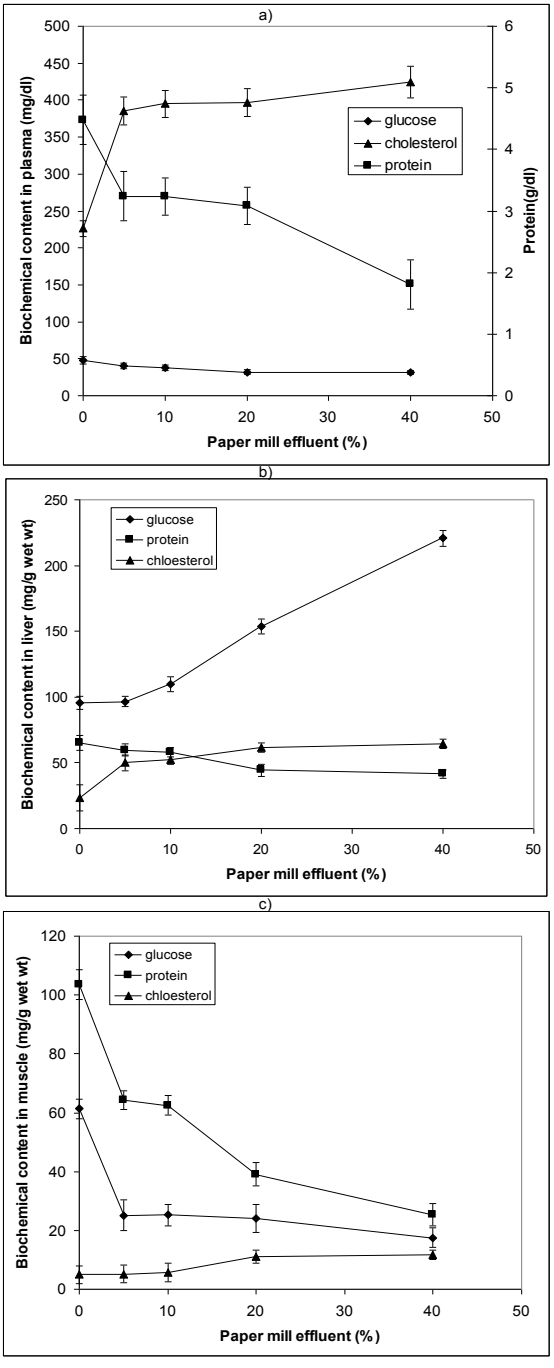

Figure 5: Biochemical content (glucose, protein and cholesterol) after exposure to paper mill effluent (a) in plasma, (b) liver and (c) muscle.

Discussion

Metal accumulation in tissues

The major objective of this study was to assess the accumulation and distribution of heavy metals (Zn, Cu, Cd, and Pb) in different tissues (gill, liver, ovary, and muscle) and its effect on hematological and biochemical parameters of *A. testudineus* exposed to paper mill effluent to understand the toxic effect. Amongst the heavy metals included in our study Zn and Cu are two important essential metals required for the normal cellular metabolism of the fish. This indicates that entry of these metals will follow the normal route of uptake when the fish are exposed to the effluent contaminated with such pollutants. In the case of fish the uptake of metal ions occurs through three major routes namely body surface, gill, and alimentary canal [22]. In general, Zn content in the control tissue (liver, gill, muscle and ovary) was higher in comparison to the other metals investigated in this study (Figure 1-4). A high amount of Zn in comparison to other elements is typical for fish [23]. The uptake of Zn by gills has been shown to be an active process. It is generally believed that fish activity regulates Zn concentration in their muscle tissue. As a result change in ambient availability of Zn in the environment is not reflected in the tissues of fish. Accumulation of Zn induces a depressive effect on tissue respiration which is consistent

with a general deficiency of oxygen in the whole fish that could lead to possible death by hypoxia. However, incomplete regulation leads to enhanced accumulation of this element in gill and alimentary canal [24]. Our observation of very high accumulation of Zn in gill (332.9 ± 2.9 µg/g dry weight) may be a result of similar failure in regulating mechanism in the fish exposed to paper mill effluent. The accumulation and distribution of metallic element in tissues/organs depends on the route through which it enters into the body that may be linked to the feeding habits. The trend of Zn accumulation in *A. testudineus* with highest accumulation observed in gills indicates that major route of entry of this metal was gill. Interestingly, in the present study ovary has shown highest zinc accumulation factor (4.08) compared to the control amongst all the tissues investigated. This indicates that besides gill and liver, ovary was a target organ for Zn accumulation in *A. testudineus* that might affect the reproduction mechanism of the fish. Vitellogenin (VTG) is important for oocyte development, a developmental phase responsible for enormous oocyte growth, where heavy metals (contaminant products) along with the essential nutrients are taken up and stored for future embryo development. Metals can also bind to different sex-linked proteins (lipoproteins and phosphoproteins) and lipids by binding to receptor mediated uptake of VTG present in fish ovaries [25]. The major edible part of the fish is muscle. Accumulation of metal ion in this tissue would have significant effect on transfer of this metal to the human being through food chain. However the accumulation of Zn was least in muscle tissues of *A. testudineus* and is comparatively a less likely target organ for Zn.

Liver and gills are known to be target organs for Cu in fish. The Cu content of the gill among all tissues in control fish was highest reflecting the intensive biochemical processes in this organ. However, Cu accumulation pattern did not follow the same trend in effluent exposed fish. In fact, maximum amount of copper accumulation was observed in liver followed by ovary. Our observation of liver as the main organ for copper accumulation agrees with the finding of liver as the target organ in *Clarias gariepinus* exposed to sub lethal levels of tannery effluent [2]. Similar result has also been reported by Moiseenko and Kudryavtseva [23]. An important deviation in Cu uptake in comparison to other metals was observation of a saturation effect on Cu accumulation in liver and gills beyond 20% and 10% effluent exposure respectively. Such phenomenon has been suggested to occur when the net uptake of copper at the apical membrane of the gill is equalized by transport across the basolateral membrane into the blood [26]. In another study, Nair et al. [24] have observed maximum accumulation of Zn and Cu in gills and alimentary canals of marine fish collected from Cochin, the southern coast of India.

Cadmium is an oxyphllic and sulfophillic element. It has affinity for multiple bonding in the body forming stable complexes with variety of organic compounds. In fish, cadmium has adverse effects on growth, inhibits calcium uptake in gills and alters liver function [27]. Barber and Sharma [28] observed maximum accumulation of Cd in kidney of fresh water fishes *Labeo rohita* (0.1558 mg Cd/g) and *Catla catla* (0.1606 mg Cd/g) exposed to Zn smelter effluent. In both the fish, kidney accumulated maximum amount of Cd followed by gill whereas liver and muscle contained less cadmium. However, maximum accumulation was found in gill in *Channa punctatus* (0.0899 mg Cd/g). Using blue gill (*Lepomis macrochirus*) Mount and Stephan [29] found maximum amount of Cd accumulation in kidney, liver, gill and gut but not significantly in bone and muscle. While studying Cd accumulation in *Cyprinus carpio*, Vincent and Ambrose [30] observed maximum accumulation in gill. Significant amount of zinc and cadmium accumulation was seen in the alimentary canal in *Mystus vittatus* collected from Mehadrigedda stream (Vishakhapatnam, India) [22]. In contrast to all these studies, our observation suggests that reproductive organ such as ovary tissues of *A. testudineus* was the major site of Cd accumulation.

Lead accumulation was mainly observed in liver and kidney by Moiseenko and Kudryavtseva [23]. The background concentration of Pb was very high in gills compared to other organs in our study. Maximum amount of Pb found in ovary is a rare observation. However, higher accumulation in liver as shown in Figure 4 was similar to the findings of Allen [31]. Further, lowest concentration of Pb as observed in muscle tissues of *A. testudineus* was similar to the finding of Oladimeji and Offem [32]. This might be attributed to the high growth factor of muscle tissue as growth may dilute the toxicant concentration [2].

In this study, metal accumulation in whole fish (Liver + gill+ muscle+ ovary) was in the order of Zn > Pb > Cu >Cd and tissue specific total metal accumulation (Zn + Cu+ Cd+ Pb) was gills > liver > ovary > muscle. The variation in metal accumulation in different tissues could be due to difference in permeability of the tissues that would determine the quantity of metal entering into the cells. There could be competition for the protein binding sites both at the mucosal cell level and in tissues. Another important factor that could play a significant role in determining the fate of each metal is presence of multiple metal ions together in the same medium such as the paper mill effluent. This condition is completely different form the studies on accumulation of metals performed in isolation with single metal component in the system. It is well known that when metals occur in association they can interact and the critical concentration of the toxic effects of some may be altered [33]. Possible mechanisms of metal-metal interaction include interchange of metal bound proteins, induction of metal binding proteins, and formation of compound complexes among metals. In interchange of metal-bound proteins competitions for carrier proteins may affect the transport of metal ions. Such as interaction between copper and Zn could be partly explained by competitions for binding sites on a protein carrier such as albumin in blood plasma [2].

Under metal stress several organisms synthesize a special type of protein, metallothionein (the metal binding protein) that helps in detoxification of the metal ion by binding and storing it as a constituent [34]. Metallothioneins are low molecular weight cystein rich protein that can selectively bind heavy metals [35]. Expression of metallothionein may equip several species enabling them to adapt and survive in mildly contaminated media. These proteins bind specifically to neutrally essential trace elements, such as Zn and Cu, as well as to potentially toxic metal as Cd and Hg [23]. This may be a reason for accumulation of high quantity of Zn, Cd, Cu and Pb in liver, gills and ovary of *A. testudineus*. Further, this study reveals the differences in the distribution of metal ion accumulation in different tissues of the fish *A. testudineus*. Higher quantity of metal ion (especially zinc, cadmium and lead) accumulation observed in this study for reproductive tissue such as ovary is very rare.

Hematology

Depletion in Hb, TEC, PCV and MCHC (Table 1) upon exposure to paper mill effluent indicates anaemia in *A. testudineus*. This may be attributed to the deficit in iron and its utilization in haemoglobin synthesis as observed in *H. fossilis* upon exposure to sublethal concentration of Ni [36]. Decreased iron uptake in intestinal villi which is evident from our previous work [18] led to poor oxygen transport [37]. This is in agreement with adverse affect on respiratory

metabolism due to exposure of PME on *O. mossambicus* [38]. Depletion may also be due to inhibitory effect on 5- amino, levulinate synthetase [39]. Increase in MCH, MCV suggests that anemia is macrolytic type similar to the report in *Tilapia mossambica* exposed to Cd [40]. Cadmium can compete with iron for the same binding site and thereby inhibit iron uptake. Increase in TLC indicates leucopoesis which is an adaptive response to the new environment against toxicant [41,42]. No change was observed in Hb and hematocrit in *C. carpio* during upon Cd exposure [13]. Increase in Hb and hematorit in *Salmo gairdneri* exposed to Cr [43]. Increase in Hb and hematocrit in fish in response to Zn [44] and Cd [45] has been reported. Cadmium exposure has been found to increase blood oxygen carrying capacity in rainbow trout [14]. Hemoglobin content and number of RBC showed no differences between control and Pb exposed fish *Anguilla anguilla* [46]. *C. gariepinus* did not show any difference in hematological parameters such as red cell count, Hb, and hematocrit on exposure to dietary Cu [15].

Tissue biochemistry

Fish exposed to varying concentration of PME were analyzed to study the tissue specific changes in their biochemical status. The overall result (Figure 5a-5c) showed decreased glucose level (except the liver) and protein in all tissues with increasing concentration of PME exposure. This was accompanied by concomitant increase in cholesterol level in all tissues. Hence, clearly indicating the nutritional stress experienced by the fish on exposure to the effluent. Hypoghycemia in plasma may be due to low blood cortisol level. Lower plasma glucose may be due to decrease glucose uptake across the intestinal wall [18], while increase in liver glucose level may be attributed to retardation of glycogenolysis due to decrease in glucose-6-phosphate activity. Impaired glucose homeostasis was suggested as a possible reason for reduction of blood glucose in *Salmo gairdneri* exposed to inorganic lead [47]. Hyperglycemia in liver is a case of activated carbohydrate metabolism. Impaired homeostasis could lead to decreased plasma glucose in dietry Cd exposed rainbow trout [14]. In vitro study suggests that Cd impairs basal or epinephrine stimulated glucose release from eel hepatocytes [48]. Hyperglycemia in liver seemed to be more persistent on fish exposed to a simulated metal contaning effluent from a sulphide ore smeltery [49]. Increase in plasma glucose level of *O. niloticus* induced by copper [11] may be associated with the activation of the hypothalamus-sympathetic chromaffin cell axes [50]. An increase concentration of glucose in the plasma indicates activated carbohydrate metabolism as a response in *O. mossambicus* to sublethal copper exposure [12]. Increased in plasma glucose levels in cat fish exposed to abandoned coal mines drainage may be due to glucose mobilization in response to environmental stress that eventually decreased and then return to normal levels after 28 days of exposure [51].

Significant decline in protein content was observed in *A. testudienus* suggesting intensive proteolysis or reduced protein synthesis. Damage of the tissues [18] and proteolysis may be due to elevated protease, aspartate aminotranferase and alanine aminotansferase activity [13]. Decrease in protein in sunfish population exposed to coal ash effluent could be due to nuitritional stress [2]. Vijayram and Vasugi [52] reported depletion in protein level in tissues of *Rasbora daniconius* on exposure to paper mill effluent, whereas Singh et al. [53] reported hypoproteinemia in fish *Heteropneustes fossilis* treated with tannery effluent. This may be due to the reduced amino acid absorption in intestine [54]. Depletion of protein and glucose and increased cholesterol level in liver was observed in *H. fossilis* exposed to nickel [36].

Cholesterol level can be used as indicator of feeding and nutritional status [2]. Our result showed increased cholesterol content upon exposure to PME in *A. testudienus*. Similar report of increase in cholesterol followed by metal exposure has been reported in *Oreochromis niloticus* [55]. Plasma cholesterol level increased after acute and chronic exposure to mercury in *Ophiocephalus punctatus* [56]. Increase in cholesterol may be due to impair regulation of liver function [18] and as a mechanism to sequester lipophilic xenobiotics [57].

Overall impact of metal accumulation on the fish metabolism

The current results indicate that metal ions present in the Paper Mill Effluent enter into the fish through gills. Thereafter, it would enter into the blood and gets transported through the blood to various organs such as liver, muscle and ovary. The highest metal accumulation factor observed for the metal ions zinc, copper and lead indicates ovary as the target organ for all these heavy metals which would ultimately affect reproductive process. Damage to the liver and muscles due to increased accumulation of metal ions would adversely affect the detoxification process causing increased toxicity and degradation of muscles leading to protein depletion. In case of cadmium, the amount of metal ions accumulated was highest in ovary, although the accumulation factor was marginally lower than muscle tissue. Accumulation of cadmium preventing iron uptake could cause anemia [41,42] which has been observed in this study. Reduced supply of oxygen would adversely affect the central metabolic pathway including TCA cycle, glycolysis and gluconeogenesis leading to shortage of energy and slowing down conversion of intermediates to other building blocks thereby affecting glucose, cholesterol and protein metabolism.

Conclusion

Exposure to paper mill effluent (PME) resulted in significant accumulation of metal ions in different tissues of *Anabas testudienus*. Accumulation of metal ions in gills established this as a major route for entry of metal ion. Zinc was accumulated highest in the fish and cadmium the lowest. Interestingly, besides gills and liver, ovary was found to be a target organ for metal accumulation with highest maximum accumulation factor. Metal accumulation leading to anemia was evident with decrease in hemoglobin, TEC, PCV and MCHC indicating impaired metal homeostasis. Decrease protein content due to damage of the muscle tissue showed 75% of the protein was lost after exposure to PME. Increase in cholesterol content in all the tissues indicates metal exposure through PME leading to loss of regulation in liver function. The overall result indicates that heavy metal present in the paper mill effluent leading to its accumulation in various tissues following the exposure could cause toxicity to *A. testudienus* and therefore may be harmful for human consumption.

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