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Effects of Bleached Kraft Pulp and Paper Mill Effluents (BKME) on the Biochemical and Hematological Parameters of Fish *Channapunctatus*

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Abstract: In this study the effects of bleached kraft pulp and paper mill effluents on the biochemical and physiological parameters of fish *Channa punctatus* were reported. Water samples were collected from River Aami. Fishes were treated with different sub-lethal doses of water samples, it shows significant alterations in level of total protein, total free amino acids, glycogen, nucleic acids and the activity of enzymes protease and transaminases, the differential leukocyte counts also showed slight differences, fewer lymphocytes and more granulocytes were found. The morphology of red blood cells had more elongate erythrocytes with a longer major axis and a shorter minor axis.

Key words: Pulp And Paper Mill Effluents · River Aami · *Channapunctatus* · Biochemistry · Hematology

INTRODUCTION

Water pollution has many sources, the most polluting of them are the industrial wastes discharged into the rivers [1-3]. The paper and pulp industry is one of the oldest industries in our country.

The pulp and paper industry world-wide produces varying amount of effluents, which are discharged into the aquatic environment. More recently, research efforts have focused on the environmental fate and effects of bleached kraft mill effluent (BKME) on a wide range of species and ecosystems. Most researches into the biological impact of pulp effluent in both field and experimental stream exposures has involved aquatic organisms, including benthic organisms [4], fin fish [5-7], white sucker [8], rainbow trout [9] and largemouth bass [10].

Chlorophenols, fatty acids and resin acids are the main acutely toxic and bioaccumulating compounds in bleached kraft mill effluent. BKME has a wide range of physiological and biochemical effects in fish living in contaminated water areas. The immunological response of fishes is impaired also due to BKME. Exposure of fish to sub-lethal concentrations of contaminants may impose considerable physiological and biochemical stress, resulting in a number of manifestations such as reduced growth, impaired reproduction, predisposition to disease, reduced locomotory and predatory performance or reduced capacity to tolerate subsequent stress [11]. Jokinen *et al*. [12] found that, in immunized roach, the antibody secreting cell response was lower and antibody titers increased more slowly in exposed fish indicating a weakened response to the antigen after sub-chronic exposure of roach to BKME. Jeney *et al*. [13] found decreased lymphocyte numbers in blood of roach collected from contaminated Lake Vatia when compared with roach from uncontaminated Lake Peurunka.

The purpose of the present study was to describe the physiological and biochemical changes in freshwater fish *Channapunctatus* caused by BKME.

MATERIALS AND METHODS

Collection of Experimental Animal: Fish *Channapunctatus* (29.21±1.83 g weight and 14.5±1.20 cm length) were collected from the Ramgarh Lake of Gorakhpur district. The collected fishes were maintained in glass aquaria containing 100 L de-chlorinated tap water

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for acclimatization to laboratory conditions for 1 week. The water in aquaria was aerated continuously. The dead animals were removed from the aquaria to avoid any contamination.

Description of Paper Mill Plant: A pulp and paper mill namely Rayana Paper Board Industries Ltd situated at Khalilabad, an industrial area of Gorakhpur region, discharges its effluents into River Aami. This pulp and paper mill manufacture writing and craft wrapping paper, produced about 64 tons of paper/day.

Collection of Water Samples: Water samples were collected from three sampling sites

Site- I:From the entry point of the effluents into River Aami
Site- II: 200 meters downstream from the site I
Site- III:200 meters upstream from the site I

Samples will be collected in early hour of day from these sites.

Biochemical Experiment: The biochemical experiments were performed by the method of Tripathi and Singh [14] and conducted in freshwater ponds, 29.28 m^3 in area and 9.19 m^3 in water volume. Pond was stocked with 60 fishes with a size difference not greater than 1.5 times [15] and then treated with different sub-lethal doses of water samples (Table 1). Fishes of control group were kept in similar conditions without any treatment. After completion of 96 hrs the muscle and liver tissue in fish *Channapunctatus* of treated as well as control group were quickly dissected out and used for biochemical estimations.

Protein: Protein levels were estimated according to the method of Lowry *et al*. [16] using bovine serum albumin as standard. Homogenates (10 mg/ml, w/v) were prepared in 10% TCA.

Total Free Amino Acids: Estimation of total free amino acid was made according to the method of Spices [17]. Homogenates (10 mg/ml, w/v) were prepared in 95% ethanol, centrifuged at 6000 xg and used for amino acid estimation.

Nucleic Acids: Estimation of nucleic acids (DNA and RNA) was performed, by methods of Schneider [18] using diphenylamine and orcinol reagents, respectively.

Table 1: Doses used for biochemical and hematological studies on fish *Channapunctatus*

Site	Type of Effluent	Percent dilution
Biochemical Studies		
Site I	BKME	9.5%
Site II	BKME	10.5%
Site III	BKME	15.5%
Hematological Studies		
Site I	BKME	5.5%
Site II	BKME	5.5%
Site III	BKME	8.5%

Homogenates (1 mg/ml, w/v) were prepared in 5% TCA at 900 C, centrifuged at 5000 xg for 20 min and supernatant was prepared and used for estimation. Both DNA and RNA have been expressed as µg/ mg tissue.

Glycogen: Glycogen was estimated by the Anthrone method of Van Der Vies, [19]. In the present experiment 50 mg of tissue were homogenates with 5 ml of cold 5% TCA. The homogenate was filtered and 1.0 ml of filtrate was used for assay.

Protease: Protease activity was estimated by the method of Moore and Stein[20]. Homogenate (50 mg/ml, w/v) was prepared in cold distilled water. Optical density was measured at 570 nm. The enzyme activity was expressed in µmol of tyrosine equivalent/mg protein/h.

Transaminases: Glutamic oxalic transaminase (AST) and Glutamic pyruvic transaminase (ALT) activities were measured according to Reitman and Frankel [21]. Homogenates (100 mg/ml, w/v) were prepared in phosphate buffer for 5 minutes and centrifuged at 1000 g for 15 minutes and supernatant was kept for estimation of enzyme activity.

Hematological Experiment: Hematological experiment was conducted in freshwater ponds, 29.28 m^3 in area and 9.19 m^3 in water volume. Each pond was stocked with 20 adult *Channapunctatus* and then treated with different sub-lethal doses (Table 2). No artificial feeding was carried out. All fish were examined after three weeks exposure. This parameter was measured according to Lehmann *et al*. [22].

Approximately 10µl of blood from each fish were used to make the blood smears. The smears were stained in May-Grunwald and Giemsa solutions. The major and the minor axes of 500 erythrocytes on each smear were measured by means of a light microscope (1000×magnification). The ratio of the major/minor axes plus the surface area of the erythrocytes were calculated. Two hundred leucocytes on each smear were determined.

Table 2: Levels of total protein, total free amino acids, nucleic acid (DNA and RNA), glycogen, protease and transaminases (AST & ALT) in liver and muscle tissue of *Channapunctatus* after sub-lethal doses of bleached effluent collected from site I, II and III after 96h

Parameter	Tissue	Control	Site I	Site II	Site III
Protein (µg/ mg)	Liver	136±0.01 (100)	76.5±0.01* (56)	83.8±0.01* (62)	130±0.02* (95)
	Muscle	154±0.01 (100)	82±0.005* (53)	90±0.003* (58)	143±0.01* (92)
Amino acid (µg/ mg)	Liver	5.5±0.02 (100)	14.0±0.01* (255)	11.5±0.02* (209)	5.8±0.01* (105)
	Muscle	7.5±0.005 (100)	15.0±0.01* (200)	13.5±0.01* (180)	8.1±0.002* (108)
DNA (µg/ mg)	Liver	27±0.002 (100)	14±0.004* (52)	15.3 ±0.02* (57)	26±0.02* (96)
	Muscle	23.5±0.01 (100)	9.4±0.005* (40)	12±0.01* (51)	22±0.001* (94)
RNA (µg/ mg)	Liver	30.5±0.01 (100)	14.8±0.02* (48)	16.3 ±0.006* (53)	28.9±0.003* (95)
	Muscle	27.2±0.005 (100)	10.8±0.01* (40)	13.2±0.005* (49)	25.5±0.002* (93)
Glycogen (mg glycogen/g of tissue)	Liver	4.41±0.01 (100)	3.1±0.01* (70)	3.5±0.01* (79)	4.3±0.005* (97)
	Muscle	3.4±0.01 (100)	2.1±0.003* (62)	2.30±0.01* (68)	3.4±0.001* (94)
Protease (µmoles pyruvate/g tissue)	Liver	0.968±0.001 (100)	1.37±0.003* (141)	1.13±0.005* (116)	1.03±0.002* (106)
	Muscle	0.812±0.002 (100)	1.28±0.01* (157)	1.16±0.01* (142)	104±0.002* (128)
(AST) (µmoles pyruvate/mg protein/hr)	Liver	5.82±0.004 (100)	6.66±0.02* (114)	6.46±0.01* (110)	6.0±0.002* (103)
	Muscle	5.22±0.01 (100)	5.68±0.01* (108)	5.45±0.004* (104)	5.35±0.01* (102)
(ALT) (µmoles pyruvate/mg protein/hr)	Liver	6.24±0.003 (100)	11.6±0.01* (186)	11.3±0.01* (181)	6.5±0.003* (106)
	Muscle	5.78±0.01 (100)	6.26±0.003* (108)	6.08±0.01* (105)	5.82±0.002* (101)

• Values are mean ± SE of six replicates.

• Values in parentheses are% change with control taken as 100%.

• Data were analyzed through student's't' test.

• *Significant ($P<0.05$), when treated groups were compared with controls.

RESULTS

Biochemical Experiment: Sub-lethal doses of water samples collected from site I and site II caused significant alterations in the level of total protein, total free amino acids, glycogen and nucleic acids and activity of enzyme protease and transaminases in liver and muscle tissue of fish *Channapunctatus*. Total protein level was reduced to 56% and 53% of control, Nucleic acid level such as DNA level was reduced to 52% and 40% of control, similarly, RNA level was reduced to 48% and 40% of control, Glycogen level was reduced to 70% and 62% of control, while Total free amino acid level was induced to 255% and 200% of control, respectively in liver and muscle tissues. The activity of transaminases Glutamic oxalic transaminase (AST) was increased up to 114% and 108% of control, whereas Glutamic pyruvic transaminase (ALT) level was increased up to 186% and 108% of control, Protease activity was significantly increased 141% and 157% of control respectively in liver and muscle tissues of fish after 96h exposure to sub lethal dose of effluent collected from **Site I** (Table 2). Similar trend of result were observed for site II (Table 2).

The value of all biochemical parameters of site III is nearly same to control group, so it is clear that the water sample collected from site III is less toxic than site I and site II.

Hematological Experiment

Hematological study is important for toxicological research, environmental monitoring of fish and their health conditions during culture because fish generally are so intimately associated with the aquatic environment. On the exposure of bleached effluent after three weeks, Lymphocyte value was reduced, monocyte value was nearly same and value of granulocyte total was higher than control group. The mean size of the erythrocytes were different, the major axis of erythrocytes was significantly longer, while the minor axis was significantly shorter than control groups, this means that their cells were more elongate. As a consequence, the ratio of the major and the minor axis were also different; ratio was higher than the control group. However the surface area of the cells did not differ (Table 3). The value of hematological parameters of site III is nearly to control group.

DISCUSSION

Data of biochemical observation, indicates that after exposure to 96h of fish with sub-lethal doses of water sample caused significant ($P<0.05$) decrease in the level of total protein, glycogen, nucleic acids and enhancement in total free amino acid level, protease as

Table 3: Effect on differential leucocytes count and the size of erythrocytes of *Channapunctatus* after sub-lethal doses, 5.5%dilution of site I, Site II and 8.5% dilution of site III, of bleached effluents after three weeks

Parameters	Control	Site I	Site II	Site III
Leucocytes				
Lymphocyte,%	70.53±0.006	62.83±0.007	65.16±0.005	67.22±0.006
Monocyte,%	10.15±0.005	10.31±0.006	10.02±0.005	10.08±0.002
Granulocyte total	16.72±0.007	20.26±0.008	18.96±0.007	17.22±0.005
Erythrocytes				
Major axis µm (ranges)	10.93±0.006 (9.00-17.00)	13.23±0.007 (12.00-17.00)	12.12±0.006 (10.0-16.0)	11.55±0.002 (9.0-15)
Minor axis µm (ranges)	7.43±0.005 (6.00-10.00)	7.12±0.006 (6.00-9.00)	7.28±0.006 (6.0-9.0)	7.18±0.005 (6.0-9.0)
Major axis/Minor axis (ranges)	1.47±0.002 (1.25-2.55)	1.85±0.001 (1.55-2.75)	1.66±0.002 (1.35-2.55)	1.53±0.006 (1.25-2.45)
Surface area µm (ranges)	80.21±0.008 (60.0-110.0)	81.11±0.007 (65.0-108.0)	79.06±0.007 (63.0-105.0)	80.38±0.001 (64.0-109.0)

Results are expressed as mean ±SE of sixreplicates

well as significant ($P<0.05$) enhancement in activities of transaminases (AST & ALT) in liver and muscle tissue of fish (Table 2).

The depletion of protein fraction may have been due to their degradation and possible utilization of degraded products for metabolic purposes. Depletion in tissue proteins of fishes due to low rate of protein synthesis under metallic stress was also reported by several workers [23, 24]. The enzyme protease functions in hydrolyzing proteins to free amino acids and small peptides. Increased protease activity in the body tissue corroborates the enhancement in the free amino acid level, the formation of which might be the result of protein hydrolysis in the tissues suggesting stimulation during toxic stress. Similar trend of results on protease activity were also reported by several workers in different animals as *Tilapia mossambica*, *Pilaglobosa* and various mammals, [25-28]. Increase in free amino acids level was the result of breakdown of protein for energy requirement and impaired incorporation of amino acids in protein synthesis [29]. It also attributed to lesser use of amino acids [30] and their involvement in the maintenance of an acid-base balance [31]. The decrease in the glycogen content in tissues indicates its rapid utilization by the perspective tissues as a consequence of toxic stress felt by the animals during the experiment. Inhibition in DNA synthesis might affect both protein as well as amino acid levels by decreasing the level of RNA in protein synthesis machinery [32]. Similar results were also observed by Yadav *et al*.[33]. Transaminases are released during cellular damage or lysis [34-36]. Stress conditions induce elevation in the transamination pathway [37]. Many other workers were also observed the increased transaminases activity in freshwater fishes exposed to various toxicants [38]. The increase may also due to damage of the organs resulting in increase protein and CHO metabolism as suggested by Nemcso'k *et al*. [39].

Hematological studies help in understanding the relationship of blood characteristics to the habitat and adaptability of the species to the environment. Many factors such as environmental and physiological are known to influence fish hematology; these include stress due to capturing, transportation, sampling, age and sex [40]. The decline in leucocytes count reported by Tana and Nikunen [41] is probably attributed to reduction in the number of circulating small lymphocytes [42], which may be resulted in the reduced resistance of stressed fish to diseases. Sizes of erythrocytes are increased than the control because the animal was in stress so consume large amount of O_2 (Table 3). Red blood cells are normally more elongated when they get older [43]. The red blood cell count of *Clariusgariepinus* was reported to have increased significantly by Annue *et al*. [44] when the fish was subjected to zinc treatment. They attributed the red blood cell elevation to blood cell reserve combined with cell shrinkage as a result of osmotic alteration of blood by the action of metal [45]. In fish blood, oxygen is carried in combination with hemoglobin and this is very important for survival of the fish. These results are also supported by many workers [10, 46-48].

On the basis of the present investigations it may be concluded that effluent from the pulp and paper mill industries has a profound effect on the biochemical aspects like hematological and muscle profile, with particular reference to energy metabolism in freshwater fish *Channapunctatus*.

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