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Stress Induced Biochemical Changes in the Liver of *Oreochromis mossambicus* Exposed to Pulp and Papermill Effluent

Kiran Joseph[1] and Elizabeth John[2]

[1] *Department of Zoology, Catholicate College, Pathanamthitta-689 645, Kerala, India*

[2] *Department of Zoology, St. Gregorios College, Kottarakkara-691 531, Kollam (Dist.), Kerala, India*

*E-Mail: ***kirankj007@gmail.com**

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The stress induced effect of pulp and papermill effluent in the fish, *Oreochromis mossambicus* was investigated in the present study. Pulp and papermill effluent discharged from Hindustan News Print LTD, Peruva, Kottayam (Dist), Kerala was collected from the discharging point and used for the study.

The adult *Oreochromis mossambicus* of size 15 ± 2 g were exposed to a sublethal concentrations (1/5th and 1/10th LC50 value) of the effluent for a period of 10, 20 and 30 days. biochemical parameters in the liver such as carbohydrate, protein, lipid and enzymes were analysed. Energy yielding nutrients such as carbohydrates, proteins and lipids were decreased tremendously for the study as the concentration and duration of the effluent exposure increased. Increased activity of enzymes such as GOT, GPT and LDH showed that the fish was under stress when exposed to pulp and papermill effluent. Fish exposed to papermill effluent showed reduced activity of ACP and ALP as the duration of exposure and concentration increased.

Key words: biochemical studies, liver, Oreochromis mossambicus, pulp and papermill effluent

The rapid industrial growth throughout the world is common and in India, particularly due to alarming rise in human population caused tremendous environmental pollution. The aquatic environment is severely affected by different types of chemicals which are toxic to the aquatic organisms (Kopecka *et al.,* 2006). The freshwater organisms particularly fishes are more susceptible to these pollutants, since, their habitats are confined and escape from such polluted habitats is impossible (Pathan *et al.,* 2009)

Fishes are widely used to evaluate the health of aquatic systems and their biochemical and physiological changes serve as biomarkers of environmental pollution (Lavado *et al*., 2006). Pollutants are entering into fish through five main routes: via food or non-food particles, gills, oral consumption of water and the skin. On absorption, the pollutant is carried in blood stream to either a storage point or to the liver for transformation. Pollutants reached in the liver may be stored or excreted through bile or transported to other excretory organs such as gills or kidneys for elimination (Nussev *et al.,* 2000).

Biochemical studies are good parameters which help to see the effect of pollutants on biochemical composition of vital tissue of fish. For the determination of both exposure and effect of pollutant on an organism biochemical alteration can serve as markers (Anel Joubert, 2000). The liver is considered to be a principal organ of detoxification in vertebrates and particularly in fish. It is also the potential site for lipid deposition in these animals (Freeman *et al*., 1983). Fish liver is a good indicator of aquatic environmental pollution. One of the important functions of the liver is to clean any poisons or pollutants from the blood coming from the intestine (Saleh, 1982).

Keeping the information in view, the present study has been undertaken with the following objectives such as to study the effect of pulp and paper mill effluent on estimation of Carbohydrate, Protein, Lipid, Glutamate Oxaloacetate Transaminase **(**GOT), Glutamate Pyruvate Transaminase (GPT), Lactate Dehydrogenase **(**LDH), Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP).

MATERIALS AND METHODS

For the analysis of biochemical and enzymatic parameters, fishes from control and sublethal conditions, were sacrificed after the exact periods (10, 20 and 30 days). For biochemical analysis, liver tissues was dissected out from the control and experimental fish. The tissues were weighed and centrifuged at 4,000 rpm using double distilled water. The supernatant was stored at 2^0C for analytical purpose. Colorimetric determination was performed using BTR – BIOTRONE 810(German) semi auto analyzer.

Estimation of Carbohydrate

Carbohydrate was analysed using carbohydrate test kit (AGAPPE Diagnostics, India) based on GOD – PAP methodology (Trinder, 1969). Blank is prepared by taking 1000µl working reagent. Standard is prepared by adding 1000µl working reagent and 10µl standard. Sample is prepared by mixing 1000µl working reagent and 10µl sample. Incubate at 37^0C for 10 minutes and measure the absorbance of the sample and standard against the reagent blank at 505nm.

Calculation:

$$\text{Carbohydrate concentration (mg/dl)} = \frac{\text{Absorbance of sample x 100}}{\text{Absorbance of standard}}$$

Estimation of Protein

Total protein was analysed using protein test kit (AGAPPE Diagnostics, India) based on direct biuret method (Gomall, 1949).1000µl total protein reagent serves as the blank. Standard is prepared by adding 1000µl total protein reagent and 20µl standard. Sample is prepared by mixing 1000µl reagent and 20µl sample. Incubate for 10 minutes at 37^0C. Measure the absorbance of standard and sample against reagent blank.

Calculation:

$$\text{Total protein Concentration (gm/dl)} = \frac{\text{Absorbance of sample x 6}}{\text{Absorbance of standard}}$$

Estimation of lipid

Total Lipid was analysed using lipid test kit

(AGAPPE Diagnostics, India) based on CHOD – PAP methodology (Allain, 1974). Blank is prepared by taking 1000µl working reagent. Standard is prepared by adding 1000µl working reagent and 10µl standard. Sample is prepared by mixing 1000µl working reagent and 10µl sample. Incubate at 37^{0}C for 5 minutes and measure the absorbance of he sample and standard against the reagent blank at 630nm.

Calculation:

$$\text{Lipid concentration (mg/dl)} = \frac{\text{Absorbance of sample x 200}}{\text{Absorbance of standard}}$$

Estimation of Glutamate Oxaloacetate Transaminase (GOT)

Glutamate Oxalo Transaminase was analysed with GOT test kit (AGAPPE Diagnostics, India) based on IFCC methodology (Anonymous, 1976). Working reagent is prepared by mixing GOT reagent 1 and GOT reagent 2 (1000µl) and mixed with 100µl sample. Incubate at 37^{0}C for 1 minute. Measure the change in absorbance/minute (▲OD/min.) during 3 minutes at 340nm.

Calculation:

GOT activity (U/L) = (▲OD/min.) x 1768.

Estimation of Glutamate Pyruvate Transaminase (GPT)

Glutamate Phosphate Transaminase was analysed in liver, kidney, gill, gonads and muscle with GPT test kit (AGAPPE Diagnostics, India) based on IFCC methodology (Anonymous, 1976). Working reagent is prepared by mixing GPT reagent 1 and GPT reagent 2 (1000µl) and mix with 100µl sample. Incubate at 37^{0}C for 1 minute. Measure the change in absorbance/minute (▲OD/min.) during 3 minutes at 340nm.

Calculation:

GPT activity (U/L) = (▲OD/min.) x 1768.

Estimation of Acid Phosphatase (ACP)

Acid Phosphatase was analysed in liver, kidney, gill, gonads and muscle with ACP test kit (AGAPPE Diagnostics, India) based on alpha - naphthylphosphate method (Hillman, 1971). Working reagent is prepared by dissolving one tablet citrate buffer with 2ml naphthylphosphate reagent. Add 1000 µl working reagent to 100 µl sample to analyse Total Acid Phosphatase. Incubate for 5 minutes at 37^{0}C. Measure the change in absorbance per minute (▲OD/min.) during 5 minutes.

Calculation:

Total acid phosphatase (U/L) = 750 x (▲OD/min.)

Estimation of Alkaline Phosphatase (ALP)

Alkaline Phosphatase was analysed in liver, kidney, gill, gonads and muscle with ALP test kit (AGAPPE Diagnostics, India) based on DGKC – SCE recommended procedure with Diethanolamine buffer (Klin,1972). Working reagent is prepared by reconstituting nitrophenyl phosphate reagent with the volume of diethanolamine reagent. Add 1000 µl working reagent to 20 µl sample to analyse. Incubate for 1 minute at 37^{0}C. Measure the change in absorbance per minute (▲OD/min.) during 3 minutes.

Calculation:

ALP activity (U/L) = 2750 x (▲OD/min.)

Estimation of Lactate Dehydrogenase (LDH)

LDH was analysed using LDH test kit (AGAPPE Diagnostics, India) based on SCE recommended method (Wei Bhaar, 1975). Add 1000µl working reagent with 10µl sample, mix and incubate at 37^{0}C for 1 minute. Measure the change in absorbance per minute (▲OD/min.) during 3 minutes at 340nm.

Calculation:

LDH activity (U/L) = (▲OD/min.) x 16030.

RESULTS

The effect of pulp and papermill effluent on biochemical constituents in the liver, of *Oreochromis mossambicus* was studied at two sublethal concentrations for long term exposure (10, 20 and 30 days). 1/5th LC_{50} of the effluent was 0.546% whereas 1/10th of LC_{50} of the effluent was 0.273%.

Carbohydrate

The carbohydrate level was found to be 57.86, 56.41 and 54.65mg/g during exposure period. Where as in sub lethal concentration 0.546% (1/5th LC_{50} of 96 hrs) the values were noted as 51.82, 49.61 and 47.71mg/g. When compared to control fishes the percentage of

decrease in the concentration of 0.273% was ranged between -6.28% to -10.14%, whereas in 0.546% concentration it was ranged between -16.07% to -21.56%. The carbohydrate levels in the liver of exposed fishes were decreased when compared to control. **(Table- 1).**

Proteins

The level of protein in the liver of the fish groups exposed to 0.273% (1/10th LC_{50} concentration) of effluent for 10, 20 and 30 days was found to contain 270.77, 256.28 and 241.36 mg/g. In the case of 0.546% (1/5th LC_{50} concentration) of effluent the values were noted as 246.82, 221.63 and 203.42 mg/g when exposed to 10, 20 and 30 days respectively. When compared to control, the percentage of decrease noticed in 0.273% (1/10th LC_{50} concentration) was -2.02% to -10.70%, whereas for 0.546% (1/5th LC_{50} concentration) it was from -10.68% to -24.73%. **(Table-2).**

Lipid

The lipid level in the liver of the fish groups exposed to 0.273% (1/10th LC_{50} concentration) of effluent for 10, 20 and 30 days was found to contain 9.990, 9.740 and 9.410 mg/g. In the case of 0.546% (1/5th LC_{50} concentration) of effluent the values were 9.580, 9.215 and 8.800 mg/g when exposed to 10, 20 and 30 days respectively. When compared to control, the percentage of decrease noticed in 0.273% (1/10th LC_{50} concentration) was -5.04% to -10.11%, whereas for 0.546% (1/5th LC_{50} concentration) it was from -8.94% to -15.93%. **(Table- 3).**

Glutamate oxaloacetate transaminase (GOT)

The fish groups exposed to 0.273% (1/10th LC_{50} concentration) of effluent for 10, 20 and 30 days the level of GOT in the liver was recorded as 13.26, 14.32 and 15.86 IU/min/g. In the case of 0.546% (1/5th LC_{50} concentration) of effluent the GOT values were 16.31, 18.26 and 20.24 IU/min/g on exposure to 10, 20 and 30 days respectively. When compared to control, the percentage of increase of GOT noticed in 0.273% (1/10th LC_{50} concentration) was 29.87% to 46.18%, whereas in 0.546% (1/5th LC_{50} concentration) it was from 59.75% to 86.54%. **(Table-4).**

Glutamate pyruvate transaminase (GPT)

The fish groups exposed to 0.273% (1/10th LC_{50} concentration) of effluent for 10, 20 and 30 days the GPT level in the liver was recorded as 10.99, 12.56 and 15.54 IU/min/g. In 0.546% (1/5th LC_{50} concentration) of effluent the values were 11.44, 18.21 and 24.53 IU/min/g on exposure to 10, 20 and 30 days respectively. Percentage increase over control noticed in 0.273% (1/10th LC_{50} concentration) was 9.46% to 52.50%, whereas for 0.546% (1/5th LC_{50} concentration) it was from 13.94% to 140.73%. **(Table-5).**

Lactate Dehydrogenase (LDH)

The LDH level in the liver of the fish groups exposed to 0.273% (1/10th LC_{50} concentration) of effluent for 10, 20 and 30 days was recorded as 129.20, 139.50 and 143.76 IU/min/g. In the case of 0.546% (1/5th LC_{50} concentration) of effluent the values were 131.64, 152.83 and 169.53 IU/min/g on exposure to 10, 20 and 30 days respectively. Percentage of increase noticed in 0.273% (1/10th LC_{50} concentration) was 3.96% to 15.43%, whereas in 0.546% (1/5th LC_{50} concentration) it was from 5.92% to 36.12%. **(Table- 6).**

Acid Phosphatase (ACP)

The ACP level in the liver of the fish groups exposed to 0.273% (1/10th LC_{50} concentration) of effluent for 10, 20 and 30 days was recorded as 4.86, 3.48 and 3.21 IU/min/g. In 0.546% (1/5th LC_{50} concentration) of effluent the values were 3.89, 2.34 and 2.00 IU/min/g on exposure to 10, 20 and 30 days respectively. When compared to control, the percentage of decrease noticed in 0.273% (1/10th LC_{50} concentration) was -4.89% to -36.44%, whereas for 0.546% (1/5th LC_{50} concentration) it was from -23.87% to -60.40%. **(Table-7).**

Alkaline Phosphatase (ALP)

The ALP level in the liver of the fish groups exposed to 0.273% (1/10th LC_{50} concentration) of effluent for 10, 20 and 30 days was recorded as 37.94, 35.36 and 28.58 IU/min/g. In 0.546% (1/5th LC_{50} concentration) of effluent the values were 34.47, 31.00 and 27.05 IU/min/g on exposure to 10, 20 and 30 days respectively. When compared to control, the percentage of decrease noticed in 0.273% (1/10th LC_{50} concentration) was -3.31% to -27.86%, whereas in 0.546% (1/5th LC_{50} concentration) it

was from -12.16% to -31.74%. **(Table-8).**

Table 1. The level of Carbohydrate (mg/g) in the Liver of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term).

Duration (Days)	Control (mg/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (mg/g)	%difference over control	Experiment (mg/g)	% difference over control
10	61.74±0.18	51.82±0.51	-16.07%	57.86±0.64	-6.28%
20	61.25±0.21	49.61±0.34	-19.00%	56.41±0.52	-7.90%
30	60.82±0.42	47.71±0.38	-21.56	54.65±0.48	-10.14%

Table.2-The level of Protein in the Liver (mg/g) of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term)

Duration (Days)	Control (mg/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (mg/g)	%difference over control	Experiment (mg/g)	%difference over control
10	276.34±0.55	246.82±0.75	-10.68%	270.77±0.35	-2.02%
20	267.91±0.45	221.63±0.80	-17.27%	256.28±0.60	-4.34%
30	270.27±0.52	203.42±0.50	-24.73%	241.36±0.30	-10.70

Table.3-The level of Lipid (mg/g) in the Liver of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term)

Duration (Days)	Control (mg/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (mg/g)	%difference over control	Experiment (mg/g)	%difference over control
10	10.520±0.35	9.580±0.52	-8.94%	9.990±0.48	-5.04%
20	10.501±0.45	9.215±0.32	-12.25%	9.740±0.25	-7.25%
30	10.468±0.43	8.800±0.28	-15.93%	9.410±0.17	-10.11%

Table.4-The level of GOT (IU/min/g) in the Liver of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term)

Duration (Days)	Control (IU/min/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (IU/min/g)	%difference over control	Experiment (IU/min/g)	%difference over control
10	10.21±0.15	16.31±0.18	59.75%	13.26±0.12	29.87%
20	10.52±0.14	18.26±0.20	73.54%	14.32±0.14	36.12%
30	10.85±0.10	20.24±0.15	86.54%	15.86±0.17	46.18%

Table.5-The level of GPT (IU/min/g) in the Liver of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term)

Duration (Days)	Control (IU/min/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (IU/min/g)	%difference over control	Experiment (IU/min/g)	%difference over control
10	10.04±0.25	11.44±0.43	13.94%	10.99±0.29	9.46%
20	10.20±0.31	18.21±0.48	78.53%	12.56±0.36	23.14%
30	10.19±0.37	24.53±0.51	140.73%	15.54±0.44	52.50%

Table.6-The level of LDH (IU/min/g) in the Liver of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term)

Duration (Days)	Control (IU/min/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (IU/min/g)	%difference over control	Experiment (IU/min/g)	%difference over control
10	124.28±0.54	131.64±0.68	5.92%	129.20±1.02	3.96%
20	122.13±0.61	152.83±0.71	25.14%	139.50±1.07	14.22%
30	124.54±0.57	169.53±0.92	36.12%	143.76±1.12	15.43%

Table.7-The level of ACP (IU/min/g) in the Liver of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term)

Duration (Days)	Control (IU/min/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (IU/min/g)	%difference over control	Experiment (IU/min/g)	%difference over control
10	5.11±0.12	3.89±0.20	-23.87%	4.86±0.14	-4.89%
20	5.09±0.16	2.34±0.31	-54.03%	3.48±0.17	-31.63%
30	5.05±0.19	2.00±0.25	-60.40%	3.21±0.22	-36.44%

Table.8-The level of ALP (IU/min/g) in the Liver of *Oreochromis mossambicus* exposed to two sublethal concentrations for 10, 20 and 30 days (long term)

Duration (Days)	Control (IU/min/g)	Concentration of Effluent			
		0.546% (1/5th of LC_{50})		0.273% (1/10th of LC_{50})	
		Experiment (IU/min/g)	%difference over control	Experiment (IU/min/g)	%difference over control
10	39.24±0.22	34.47±0.23	-12.16%	37.94±0.25	-3.31%
20	39.59±0.18	31.00±0.28	-21.70%	35.36±0.33	-10.68%
30	39.62±0.15	27.05±0.11	-31.74%	28.58±0.40	-27.86%

DISCUSSION

Fishes were exposed to different sub lethal concentrations for long term exposure, the biochemical parameters like Carbohydrate, Protein, Lipid, ACP and ALP showed significant decrease in relation to control. Similarly, enzymes like GOT, GPT and LDH showed acceleration and inhibition of their activities increase in effluent concentration and exposure duration.

Carbohydrate

Carbohydrates are important components of biological system. During stress condition, fish need more energy to detoxify the toxicants and to overcome

stress. In the present investigation, significant decrease in the carbohydrate content has been noticed in the liver when the exposure duration of effluent is increased. The percentage of decrease was more pronounced in fish groups exposed to higher exposure period of 1/5th concentration. The decrease in carbohydrate is mainly due to the hepatic synthesis of detoxifying enzymes requires high energy levels (Hori *et al.,* 2006).

Protein

Protein is one of the main sources of energy. The quantitative determination of total protein reflects the liver capacity of protein synthesis and denotes the osmolarity of the blood and the renal impairments. The data showed that the protein content was decreased in the liver, of the fish when fishes were exposed to long term in two sub lethal concentrations of the effluent. The decrease in protein content was dose dependent as well. The depletion in the proteins may be due to impaired or low rate of protein synthesis, their utilization in cell repair and organization and the decrease in uptake of amino acids into the polypeptide chain (Rajamanickam and Muthuswamy, 2008).

Lipid

The direction of change in the lipid parameter seems to be dependent on types of contaminants, the concentration, mode of its action, duration of exposure and fish species (Heath, 1991). The results of the present study showed a significant decrease in the lipid content of liver in both concentrations. As the concentration and exposure duration was increased, the lipid content was decreased. This may be due to increased utilization of lipid to meet additional energy requirements under stress of low oxygen take up. Similar findings are also observed by Kulkarni and Suniti Dharawadkar (1998).

Glutamate oxaloacetate transaminase (GOT)

GOT is an enzyme involved in transamination reactions and present in most of the tissues which shift metabolic cycle from amino acids to alpha keto acids which enter into Kreb's cycle and hence cause the acceleration or deceleration of the intermediary metabolism. The results of the present study showed increase in the GOT activity in the liver tissues. This may be due to decreased metabolic activity, destruction of enzyme system by blocking the active sites and tissue damage (Bhatanagar and Tyagi, 1995).

Glutamate pyruvate transaminase (GPT)

GPT is another transaminase enzyme involved in transamination reactions. GPT activity is high in liver tissues and hence the level increases markedly in acute liver disorders when the liver cells are damaged. In the present study on exposure to pulp and papermill effluent, the GPT level was increased in liver. The increase in GPT activity may indicate anaerobic nature of carbohydrate metabolism in fish, possibly to meet the increased energy demands under sustained and prolonged toxic stress as supported by Ramana Rao *et al.,* (1990).

Lactate Dehydrogenase (LDH)

LDH is an active enzyme of glucose metabolism and any effect on its activity directly affects the energy production of the cell.

The LDH activity in the fish groups exposed to the effluent showed remarkable increase in the liver tissues. As the time of exposure and concentration of effluent increased, the activity also increased. Increased activity of LDH in the tissues could be because of increased secretion by the specialized cells or by changes in the permeability of their cell membranes to stop leach out of the enzymes and keep stored in the cells as stated by Abdel-Hameid (2009).

Acid Phosphatase (ACP)

Acid phosphatases are hydrolytic lysosomal enzymes and are released by the lysosomes for the hydrolysis of foreign material and hence it has a role in certain detoxification functions (Jaroli and Sharma**,** 2005). The fishes were exposed to pulp and paper mill effluent showed remarkable decrease in the ACP activity as the days of exposure and concentration of effluent increased. This pattern of decrease in ACP activity was noticed in liver tissues. The decreased ACP activity indicates the disturbance in the structure of cell organelles, like endoplasmic reticulum and membrane transport system. Such damage to cell organelles has been reported by (Nchumbeni *et al*., 2007).

Alkaline phosphatase (ALP)

ALP is composed of several isoenzymes that are present in practically all tissues of the body, especially in cell membranes. In the present study, the liver tissues of the effluent exposed fish, the ALP level was found to be reduced. This reduction was time dependent. The ACP level was decreased in $1/5^{th}$ concentration when compaired to $1/10^{th}$ concentration of LC_{50}. The decrease in the ALP level in the present study may be attributed to the accumulation of toxicants in the tissues of the fish (Humtsoe *et al*., 2007), which interfere the synthesis of enzyme protein and increased metabolism due to an increase of toxic substances and the production of toxic metabolic products destructive to the enzymes.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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