

VOLUME 3

ISSUE 2

2023

ISSN 2583-1690



**INTERNATIONAL
JOURNAL OF
BIOLOGICAL AND
ENVIRONMENTAL
INVESTIGATIONS**

Forum for Biological and Environmental Sciences
Published by Saran Publications, India



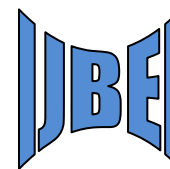
International Journal of Biological and Environmental Investigations

Contents available at Journals Home Page: www.ijbei.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Managing Editors: Dr. Abhishek Kumar & Dr. Abhai K. Srivastava

Published by: Saran Publications, Gorakhpur, India



ISSN: 2583-1690

Eco-toxicological Impact of Pulp and Paper Mill Effluent on the Behaviour and Whole Body Respiration of a Fresh Water Fish *In Vivo*

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Received: 22nd October, 2023; Accepted: 19th November, 2023; Published online: 4th December, 2023

<https://doi.org/10.33745/ijbei.2023.v03i02.002>

Abstract: The objective of this study was to assess the impact of Pulp and Paper Mill effluent on the behavior and respiratory physiology of fresh water fish under laboratory controlled conditions. Effluent exposed fish under laboratory experimental conditions showed symptoms of heavy metal toxicity. Experiments were conducted at maximum allowable concentration of the effluent waste. Decrease in body weight, brain somatic index (BSI), liver somatic index (LSI) and whole body respiration rate of the effluent exposed fish was observed compared to control fish. The effluent exposed fishes were allowed to recover in pollutant free normal water medium. Partial recovery in early period and no recovery at higher exposure period were observed. The observed effects were only due to heavy metal accumulation in the fish body absorbed from the effluent contaminated aquarium water and fish collected from the effluent contaminated crop fields. The behavior and impact observed in the experimental fish was almost similar to the fishes collected from contaminated crop fields.

Keywords: Effluent, Heavy metal, Toxicology, Fish, Brain somatic index, Liver somatic index, Body weight, Respiration

Citation: Tripathy A.P. and Panigrahi A.K.: Eco-toxicological impact of pulp and paper mill effluent on the behaviour and whole body respiration of a fresh water fish *in vivo*. Intern. J. Biol. Environ. Invest. 3(2): 9-18, 2023.

<https://doi.org/10.33745/ijbei.2023.v03i02.002>



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Introduction

Pulp and Paper mills are the major polluters of aquatic environments. These industries add toxic heavy metals like mercury, cadmium, lead and other heavy metals into the environment, polluting nearby water bodies and affecting aquatic flora and fauna. Chmielowska-Bak *et al.* (2021) cautioned the adverse effect of metals on the

environment and their impact on plant performance. The paper mill effluents are discharged from the industry into the nearby water body (Iqbal *et al.*, 2013) after physical and chemical treatments (Dey *et al.*, 2018; Tripathy *et al.*, 2022) indicating the need of proper biological treatment. Dixit *et al.* (2018) reported presence of heavy metals in the final discharged International

Paper mill effluent even after so called biological treatment. Tripathy *et al.* (2022) reported that the effluent of the paper mill severely affected the flora in and around the effluent canal and impacted the water quality of the river. These authors also indicated that usage of this contaminated river water to irrigate the crop fields will have far reaching consequences (Tripathy and Panigrahi, 2023). Waste generation by any industry and the quality of waste depends on the technology and adopted treatment protocol of any Pulp and Paper industry. It is not possible to eliminate waste generation by the system (Kaur *et al.*, 2021) but cleaner and environment friendly technology can be used for protection of natural water bodies. The present study was planned to test the impact of Paper mill effluent on the behavior and respiratory physiology of a fresh water fish under laboratory controlled conditions.

Materials and Methods

Test chemical:

JK Pulp and Paper Mills, Jaykaypur, Raygada, Odisha is located at latitude 19.247°N and longitude 83.409°E and its effluent waste meets the River Nagavalli at 19.252°N and 83.420°E. Samples from the mid effluent canal were collected in plastic containers and brought to the laboratory for analyses.

Maintenance of fishes:

Oreochromis mossambicus (Peters 1852) of medium size (15-20 g) were collected from the local nursery. The fish were acclimatized under laboratory conditions for 20 days before starting the experiment. The fish were maintained in aquarium of 60 x 80 cm, containing 50 L of water. Chlorine-free tap water was used in both control and experimental aquaria. The water was changed daily. Air was bubbled through water of the aquarium to maintain the dissolved oxygen at 85±5% air saturation value. The physico-chemical quality of the water of both control and exposed aquaria were measured during the experimental period (APHA, 1974) and maintained at the same level. Living uncontaminated earthworms from

garden showing no signs of contamination were collected and fed daily to both control and exposed fish initially and slowly the diet was changed to toxicant-free chopped goat liver and after few days given small slices of boiled eggs during holding and throughout the experimental exposure and recovery period. After acclimatization, the fish were washed thoroughly with 1% dilute Potassium permanganate (KMnO₄) solution, so as to prevent any infection. The test solution of the experimental aquarium was changed daily so as to maintain the constancy of the heavy metal concentration. The experimental aquarium was washed thoroughly to remove any amount of heavy metal adhered to inner glass surface. Exposed fish were observed daily to record any change in behavior, when compared to control fish.

Physico-chemical analysis of effluent was conducted by following the procedure of APHA (1998). The report of the effluent study was presented by Tripathy *et al.* (2022 a, b). The Dissolved oxygen was measured by modified Winkler's method. Effluent was brought in glass containers and stored in cold room for use in laboratory experimental work. Live fish respiration rate studies were conducted on the spot following the Winkler's method of dissolved oxygen measurement (APHA, 1998). Respiration rate of the fish was evaluated following the procedure as suggested by Panigrahi and Misra (1980). Experimental studies were conducted in the laboratory by taking the Paper mill effluent and *Tilapia* (*Oreochromis mossambicus*, Peters) fish. Graded series of concentrations of the freshly collected Paper mill effluent waste was prepared in 2L jars and *Tilapia* fish (small size) was introduced in each jar to grow for a period of 30 days. Fish was observed daily for change in behavior and mortality rate was recorded. The exposed jars were also tested for toxicity studies in temperature control room for the same period to study the impact of temperature stress. Fish body weight was measured by top pan balance, brain (BSI) and liver (LSI) somatic index was calculated by measuring the weight of brain and

liver tissues dissected and weighed carefully. Feeding of the control and effluent exposed fish was only once in a day with minced boiled egg (Panigrahi and Misra, 1978). The whole animal oxygen uptake rates of the test and control fish were measured using five wide mouth 2 liters capacity flasks. Each flask containing the test solution and a test fish was hermetically sealed. A reference flask was kept without fish to check any change of oxygen concentration during the experiments, due to the presence of microorganisms. Any change of oxygen concentration, caused by the microorganism was computed with the final data. After 30 min, the dissolved oxygen of all experimental flasks was determined according to the modified Winkler's method (Ashby, 1973; Panigrahi, 1980). The same procedure was repeated for the control fish. The reduction of the dissolved oxygen concentration equals the amount of dissolved oxygen consumed by the fish in 30 min. The oxygen uptake was expressed as $\text{mg O}_2 \text{ g}^{-1} \text{ h}^{-1}$ (Panigrahi, 1980).

The effluent exposed fishes were transferred to toxicant free medium for recovery studies. After 7d interval experiments were conducted for change in body weight, BSI, LSI, whole body respiration up to 28d exposure. After 7 d exposure, a batch of exposed fish was transferred to toxicant free medium for 7d recovery studies daily. After 14d exposure, another batch of exposed fish was transferred to toxicant free medium for 14 d recovery studies. After 21 d exposure, a batch of exposed fish was transferred to toxicant free medium for 21 d recovery studies. After 28 d exposure, a batch of exposed fish was transferred to toxicant free medium for 28 d recovery studies. In all recovery sets, the fishes were observed for behavioral change, body weight change and change in whole body respiration to find out the possibility of recovery. Statistical analyses were carried out to test the levels of significance of observed data.

Results

The effluent mixed water is used for irrigation of the crop fields. The pH of the crop field ranged

between 7.87 to 7.95 receiving effluent mixed water from the river and the soil pH was little higher than the water pH in the crop field selected for the study. The soil and water temperature ranged between 23.4 to 24.6°C. The data indicated presence of all the three tested heavy metals in the crop field soil and water, residual heavy metals in rice crop plant leaves absorbed from the crop fields and in the algal scum collected from the crop fields. The farmers use Paper mill effluent contaminated Nagavalli river water for irrigation of crop fields at regular intervals. The source of heavy metals in the crop fields being the water used for irrigation from the Nagavalli River. In crop field, significant amount of cadmium, lead and mercury was available in the crop field soil to the extent of 81.5 ± 9.8 , 12.5 ± 1.5 and 83.2 ± 14.6 $\mu\text{g/g}$ dry weight, respectively. In crop field significant amount of cadmium, lead and mercury was available in the crop field water to the extent of 53.8 ± 11.4 , 8.8 ± 2.6 and 48.6 ± 8.8 $\mu\text{g/l}$, respectively.

Initially the effluent waste exposed fish appeared lethargic for the first 2-3 days then became normal. Activity of the exposed fish decreased with the increase in exposure period. After 21 day of exposure, feeding decreased and opercular movement decreased significantly. Towards end of the exposure period fish death started at higher effluent concentrations. Death of fishes started first in higher effluent concentrations and no death was recorded in the control set. Control fish remained clinically healthy throughout the experimental period. Fish death was recorded daily and lethal concentration values were computed. The maximum allowable concentration (MAC value) value was 1.86% effluent concentration. Higher lethal concentrations values were observed with the increase in effluent concentration. Temperature stress study indicated that the fish could resist higher effluent concentration at 5°C lower from normal temperature. But 10°C lower, 5°C and 10 °C higher temperature increased the stress and the fish could only survive at lower effluent concentrations (Table 1).

Table 1: Lethal concentration values of the effluent on a fresh water fish under laboratory controlled conditions for 30 days of exposure and impact of temperature stress on the lethal concentration values

Lethal concentration (LC) values, 30 days of exposure	Paper Mill effluent (containing Hg, Pb and Cd) ml / liter				
	18°C (-10°C)	23°C (-5°C)	28°C Normal	33°C (+5°C)	38°C (+10°C)
LC ₀₀ (X) -MAC	1.74	1.94	1.86	1.64	0.53
LC ₅₀ (Y)	2.09	2.85	2.34	2.18	0.88
LC ₉₀ (Z)	3.95	4.28	4.15	3.54	3.44
LC ₁₀₀	7.48	8.61	8.42	7.28	6.36

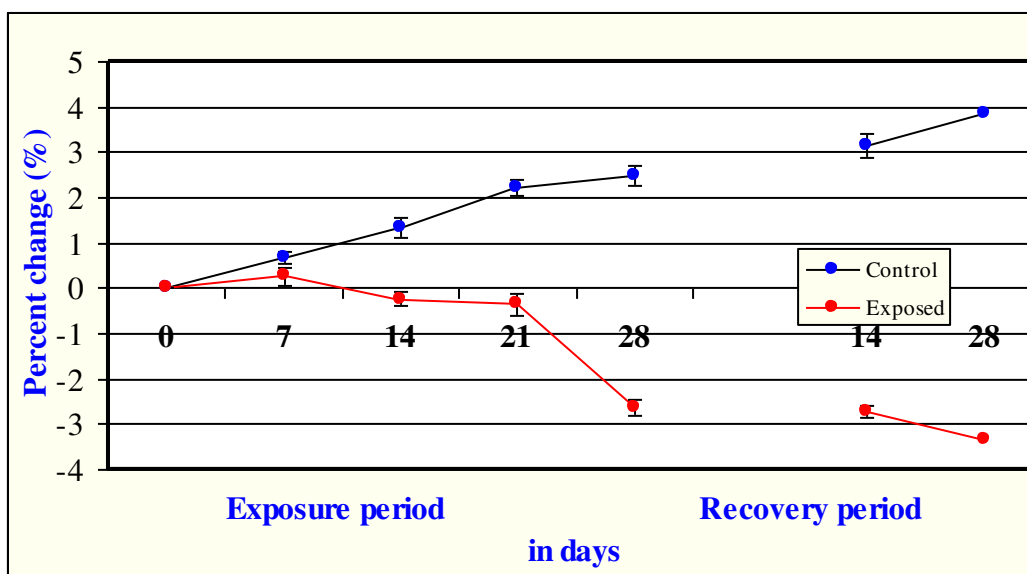


Fig. 1: Per cent changes in body weight of the control and effluent exposed Tilapia fish at different exposure and recovery periods.

Control fish showed normal growth rate as per feed provided during the experimental study. Excess feeding was avoided during the experimental period. The control fish showed 2.48% increase in body weight after 28 days in the jars. Whereas 2.64% decrease was noted in 28 days effluent exposed fish. The exposed fish showed 5.12% decrease in body weight when compared to control fish. The body weight of effluent exposed fish depleted with the increase in exposure period. The exposed fish was shifted to effluent free medium, where no recovery was noted, rather exposed fishes showed further depletion in body weight during recovery period. The 28 days exposed fish could not recover to normalcy even after 28 days of recovery and 7.2% decrease in body weight was noted on 28 days of recovery (Fig. 1). The brain somatic index and

liver somatic index decreased with the increase in effluent exposure period (Fig. 2).

No significant change in brain somatic index (BSI) was observed in the effluent exposed fish when compared to control fish BSI till 14 days exposure period. With the increase in exposure period the BSI decreased significantly. No recovery in BSI was observed in the effluent exposed fish during recovery period. The brain somatic index of the effluent exposed fish further depleted during the recovery period when compared to 28 days exposure value. Liver Somatic Index (LSI) decreased significantly in effluent exposed fishes and the impact of the effluent was drastic on the exposed fishes (Fig. 2). The LSI decreased from 1.98 ± 0.15 to 1.14 ± 0.08 on 28 days exposure and from 1.97 ± 0.06 to 0.74 ± 0.07 on 28 days of recovery, showing no

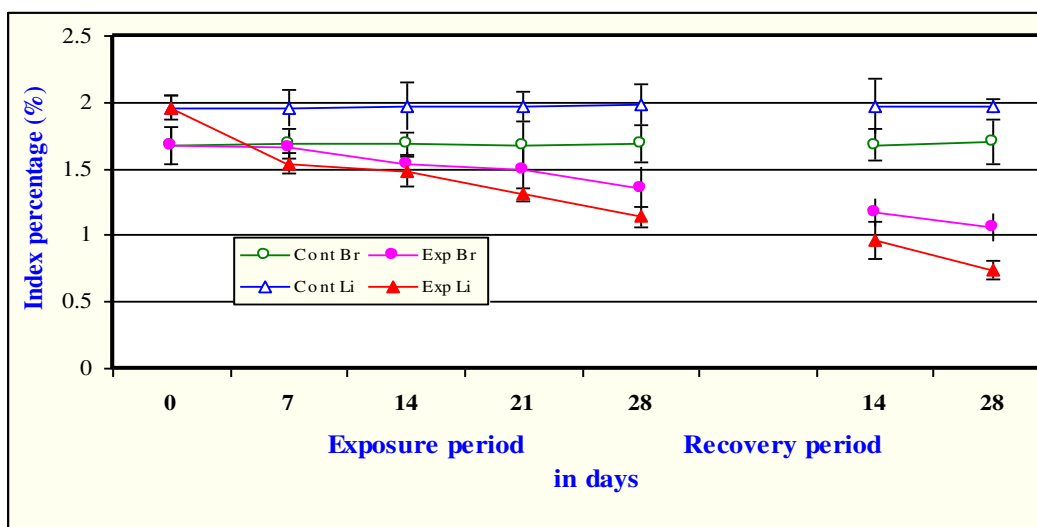


Fig. 2: Changes in BSI and LSI in control and effluent exposed Tilapia fish at different exposure and recovery periods.

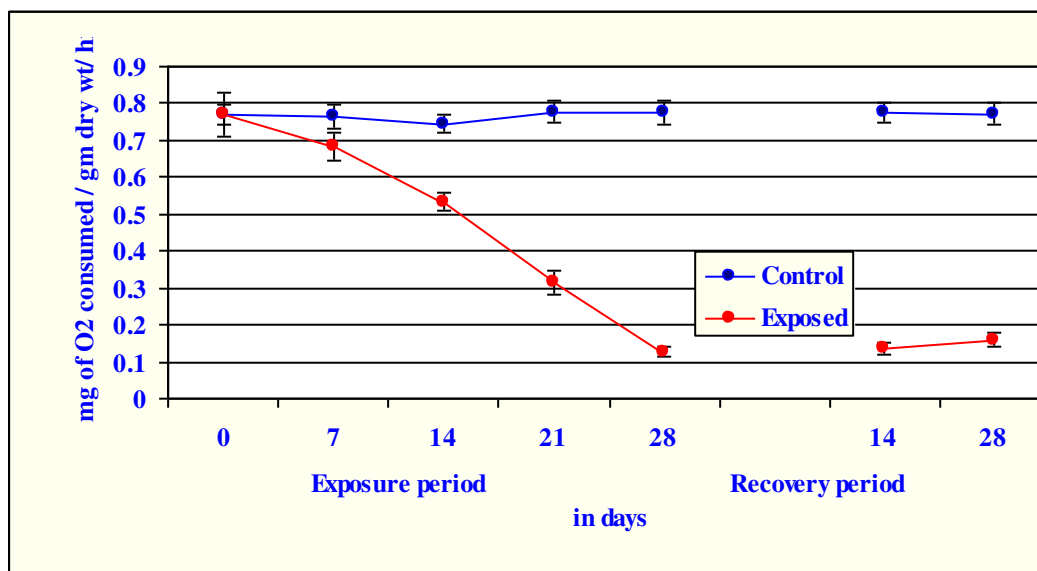


Fig. 3: Changes in whole body oxygen uptake of control and effluent exposed fish under laboratory controlled conditions at different exposure and recovery periods.

recovery. The whole body oxygen uptake of the normal uncontaminated fish was maintained within 0.764 ± 0.034 mg of O₂ consumed/g dry wt/h to 0.778 ± 0.029 mg of O₂ consumed /g dry wt/h during the entire period of experimentation (Fig. 3). The whole body oxygen uptake depleted from 0.745 ± 0.024 mg of O₂ consumed /g dry wt/h to 0.534 ± 0.027 mg of O₂ consumed /g dry wt/h on 14th day of exposure showing 28.3% decrease

over the control value and from 0.775 ± 0.031 mg of O₂ consumed /g dwt / hr to 0.126 ± 0.014 mg of O₂ consumed /g dry wt/h on 28 days of exposure showing 83.7% decrease over control value (Fig. 3). During recovery studies insignificant increase in whole body respiration was noted compared to 28 days exposed value. Recovery by 1.06% on 14th day and 4.3% decrease on 28 days of recovery compared to 28 days exposed value was

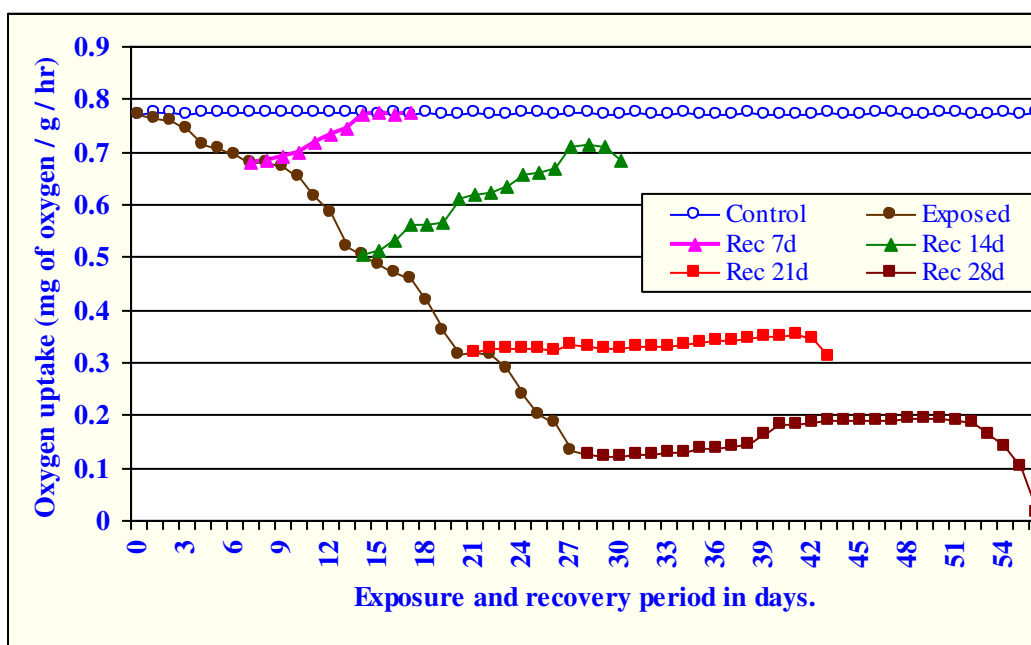


Fig. 4: Changes in whole body oxygen uptake in control and effluent exposed fish. Changes in whole body oxygen uptake in recovery period when compared to control (Exposure period = recovery period).

insignificant. The next set was planned to study percentage of recovery possibility of the effluent exposed fish transferred to effluent free normal water where the period of exposure was same with that of recovery period. The whole body respiration rate (WBRR) remained almost in the same range from 0.771 ± 0.012 to 0.775 ± 0.034 mg of O_2 consumed /g dry weight/h during the entire 56 days of experimentation. The effluent exposed fish respiration rate (oxygen consumption rate) decreased from 0.772 ± 0.025 mg of O_2 consumed /g dry wt/h to 0.681 ± 0.022 mg of O_2 consumed/g dry wt/h after 7 days of exposure showing 11.79% decrease over the control value. The 7 days effluent exposed fish was shifted to effluent free medium for recovery studies. The exposed fish interestingly recovered showing positive signs and within 7 days the effluent exposed recovered to its pre-test activity. The respiration rate increased from 0.681 ± 0.022 mg of O_2 consumed/g dry wt/h to 0.773 ± 0.015 mg of O_2 consumed/g dry wt/h, a normal control value range (Fig. 4). The behavior of effluent exposed fish after recovery was normal. The respiration rate of effluent exposed fish further depleted from 0.773 ± 0.017 mg of O_2

consumed/g dry wt/h to 0.504 ± 0.031 mg of O_2 consumed/g dry wt/h after 14 days of exposure showing 34.8% decrease over the respective control value. The 14 days effluent exposed fish was transferred to effluent free medium for recovery studies. The exposed fish interestingly recovered showing positive signs and within 13 days the effluent exposed fish recovered. The respiration rate increased from 0.504 ± 0.031 mg of O_2 consumed/g dry wt/h to 0.714 ± 0.029 mg of O_2 consumed/g dry wt/h. The behavior of effluent exposed fish after recovery was normal but when the fish was allowed further recovery for few more days, the 14 days exposed fishes started dying (Fig. 4). The respiration rate of effluent exposed fish further depleted from 0.774 ± 0.011 mg of O_2 consumed/g dry wt/h to 0.318 ± 0.017 mg of O_2 consumed/g dry wt/h after 21 days of exposure showing 58.9% decrease over the respective control value. The 21 days effluent exposed fish was transferred to effluent free medium for recovery studies. The effluent exposed fish interestingly could not recover to its pre-test activity. The exposed fish survived but the respiration rate value was almost in the same

range without any recovery. The behavior of 21 days effluent exposed fish after 21 days recovery was not normal and when the fish was allowed further recovery for few more days, the 21d exposed fishes died (Fig.4). The respiration rate of effluent exposed fish further depleted from 0.775 ± 0.027 mg of O_2 consumed/g dry wt/h to 0.124 ± 0.009 mg of O_2 consumed/g dry wt/h after 28 days of exposure showing 84% decrease over the control value. The 28 days effluent exposed fish was transferred to effluent free medium for recovery studies. The effluent exposed fish interestingly could not recover to its pre-test activity. The exposed fish survived but the respiration rate value was almost in the same range without any recovery. The behaviour of 28 days effluent exposed fish after 28 days recovery was not at all normal and fishes died gradually (Fig. 4). From the observed data it can be inferred that when fishes were exposed to short durations, the possibility of effluent exposed fish to recover and the recovery fish behaved just like normal uncontaminated fish. But if the exposure period is prolonged then the chances of recovery was questionable and not certain. At higher recovery period, most of the exposed fish died indicating permanent damage to the metabolic systems of the effluent exposed fish. The respiration rate of the effluent exposed fishes declined steadily showing a negative significant correlation. The above data indicated that the effluent is deadly toxic and adversely affects the fresh water fishes inhabiting in crop fields. This observation confirms fish death at the contaminated sites of Chandilli village near JayKaypur, Rayagada.

Discussion

The effluent of JK Pulp and Paper mill is dangerously toxic and disturbed the physico-chemical status of the river basin. The contaminated river was used for irrigation of crop fields located near both the sides of river. The crop fields receiving contaminated river water were seriously affected (Tripathy *et al.*, 2022a; Tripathy and Panigrahi, 2023). Tripathy *et al.* (2022a, b) reported that effluent of JK Paper mill is highly

toxic and affected the whole flora and fauna of the area. The same authors also reported that this contaminated river water contained toxic heavy metals, which were absorbed and retained by the crop, non-crop and algal flora of crop fields. The pulp and paper mill effluent pose acute and chronic effects to fish species due to the complex nature of effluent containing heavy metals and endocrine disrupting chemicals. Adequate treatment of the effluent was suggested by Dey *et al.* (2013). Patel *et al.* (2013) worked on JK Pulp and Paper mill treated effluent and mentioned that the effluent used for irrigation for some crop plants improved the soil fertility and organic carbon, increase in soil salinity and sodicity but forgot to mention about heavy metal accumulation in crop plants. The same authors suggested for irrigation of semi tolerant crops but warned that continuous use of effluent should be monitored regularly to avoid a disaster. Dey *et al.* (2018) reported that the paper mill effluent was extremely toxic even in very low concentrations to fishes. Abnormalities like hepatic and histological damage in *Cyprinus carpio* fingerlings was observed by the same authors. Long term exposure to Cd could lead to lung cancer; kidney diseases, bone damage in humans if rice from the contaminated soil is consumed (Zhang *et al.*, 2021). Fish serve as important indicator for water quality and human health assessments as they can bioaccumulate contaminants to higher magnitudes (Stahl *et al.*, 2021). Heavy metals like Hg, Pb, Cd, Cr, As, disrupt cellular events like growth, differentiation, damage-repairing and apoptosis (Balali-Mood *et al.*, 2021). These cause DNA methylation, defects in DNA repair resulting into genomic instability and carcinogenicity. Hg accumulates primarily at protein fraction in fishes. Consumption of contaminated fishes with Hg, Cd and Pb makes way to human exposure to mercury, cadmium and lead poisoning (Nong *et al.*, 2021). The study of Fernandez-Trujillo *et al.* (2021) indicated that heavy metal and metalloid pollution caused potential risk for the fishes inhabiting the freshwaters. Hg, Cd and Pb are toxic metals prone to be accumulated in fish muscles and tissues. The

accumulation of heavy metals depends upon temperature variation in the environment and acute and chronic doses vary accordingly. Increase in temperature and low pH can result in intense accumulation of Hg, whereas suppresses the penetration of Cd and Pb due to increase in salt concentration. The continuous industrialization and by different agricultural practices, different heavy metals were released to the coastal waters of Ondo state, Nigeria (Olusola and Festus, 2015), where high levels of heavy metals were found in gills and eyes of the fish tested and lowest in the muscles as the heavy metals target the metabolically active tissues. The muscle showed relatively lower metabolic activity than gills and the presence of heavy metals in water, sediment and tissues of fish species can be a serious concern for the human use. Boudjema *et al.* (2022) reported that heavy metal interactions can affect bioaccumulation and consequently toxicity. The same authors also agreed to the earlier reports that competition between chemically similar ions (Azizi *et al.*, 2018) for the binding sites can significantly affect bioaccumulation of heavy metals. This is in agreement with the present study. In addition, we are of the opinion that due to interaction of other chemicals present in the effluent waste and chemically similar heavy metal ions might have competed for absorption and might have fought for interacting with binding sites leading to less absorption and retention of heavy metals in fish body. The toxicological actions of a heavy metal depends on absorption, the amount of bioaccumulation, its abnormal interaction with an important and essential biomolecule (Pelletier *et al.*, 2004), site of action and interaction and interference in biochemical metabolic processes in plant or animal body.

In our present study, it was observed that the effluent exposed fish was affected seriously. The depletion of brain somatic index and liver somatic index indicated that the effluent can cause disorders. Significant decrease in whole body oxygen uptake indicated that the effluent of the industry is deadly toxic and the effluent exposed fish showed significant depletion in oxygen

uptake. The depletion in BSI, LSI and oxygen uptake indicated stress and the effluent of the industry is the causative agent. The exposed fish appeared lethargic and ataxia appeared on 3rd day onwards. The exposed fish stopped feeding for the first two days and later the exposed fish took normal food. Metals can become toxic when their uptake is not balanced by excretion processes and detoxifying systems (Castaldo *et al.*, 2020). The present investigation is a combined effect of effluent waste study on fishes under field and laboratory conditions. Both the observed data showed strong similarity, except that the laboratory experimental fish showed high inhibition than the crop field tested fish. The difference was probably due to the environmental impact and interaction which might have reduced the toxicity. The accumulation of heavy metals in fishes depend strongly on the exposure period, age of the fish, metabolic efficiency and the type of food they eat. Monitoring, analyzing and characterizing all types of toxicants including heavy metals in aquatic environments indicated the water resource condition, status, usability of water for drinking and agricultural purposes and protection of plants, animals and most important is environment and human health. All the paper industries should be very careful and should monitor the effluent regularly and must remove all the heavy metals present in the effluent waste by proper biological treatment by using the protocols of bioremediation, phycoremediation and phytoremediation for protection of environment, water bodies, flora and fauna of water bodies, crop field inhabiting plants and animals receiving effluent waste.

Conclusion

The Paper mill effluent is deadly toxic and discharged into water bodies without proper biological treatment as it contained significant amount of heavy metals like Hg, Cd and Pb. The contaminated river water is used for irrigation of the surrounding crop fields. The crop inhabiting organisms like crop plants, non-crop plants, algal mass, fresh water fishes, earthworms and snail

accumulated significant amount of heavy metals. The impact was severe as observed from the present study. The effluent significantly altered the BSI, LSI, and whole body fish respiration. Partial recovery at up to 14th day of exposure followed by insignificant recovery of 21d exposure was noted. No recovery at higher exposure period was marked. The effluent of the industry causes irreversible damage to the respiratory metabolism of the exposed fish. The effluent needs to be treated physically, chemically and by potent biological organisms which have shown efficiency to survive in heavy metal contaminated environments.

Acknowledgements

The authors wish to thank the authorities of GIET University, Gunupur, Odisha, India and Berhampur University, Berhampur, Odisha, India for permitting the authors to use the laboratory and library facilities.

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