FISHERIES & AQUATIC LIFE (2018) 26: 243 - 256
Archives of Polish Fisheries
DOI 10.2478/aopf-2018-0028

RESEARCH ARTICLE

Assessment of pulp and paper mill effluent quality and its toxicity to fingerlings of *Cyprinus carpio*

Sangeeta Dey, Manabendra Dutta Choudhury, Suchismita Das

Received – 27 April 2016/Accepted – 24 September 2018. Published online: 31 December 2018; ©Inland Fisheries Institute in Olsztyn, Poland
Citation: Sangeeta Dey S., Choudhury M.D., Das S. 2018 – Assessment of pulp and paper mill effluent quality and its toxicity to fingerlings of *Cyprinus carpio* – Fish. Aquat. Life 26: 243-256.

Abstract. Effluents from a pulp and paper mill in India were analyzed for various physicochemical parameters and heavy metals. Multivariate statistical analysis was used to interpret the data obtained and predict the toxicity of each component to *Cyprinus carpio* L. fingerlings. The results showed that most of these parameters exceeded recommended levels set by various agencies, and the effluent was not suitable for discharge into agricultural fields or water bodies without further treatment. The results of static renewal toxicity tests revealed median lethal concentrations at 24, 48, 72, and 96 h to be 42, 25, 18.6 and 14.5%, respectively, and the fingerlings exhibited severe behavioral anomalies. The sublethal dose of 2.9% (v/v) caused alterations in the gill and liver at durations of 7, 14, 21, and 28 d. Anomalies such as mucous secretion, leucocyte infiltration, curling of the secondary lamellae tip, clubbing, and the fusion of the secondary lamellae, aneurism, etc. were prominent. The liver showed inflammatory infiltration, cytoplasmic inclusion bodies, vacuolar degeneration, fatty degeneration, pyknosis, karyolysis, distorted pancreatic area, and piecemeal necrosis, among other pathological symptoms. The study concluded that the effluent, even in greatly diluted form, was highly toxic and the severity of responses depended on the length of exposure.

Keywords: common carp, physicochemical, metal, multivariate analysis, LC_{50}, chronic, histopathology

S. Dey, M.D. Choudhury, S. Das [✉]
Aquatic Toxicology and Remediation Laboratory,
Department of Life Science and Bioinformatics,
Assam University, Silchar, India
e-mail: drsuchismita9@gmail.com

Introduction

With more than 700 paper mills located across its length and breadth, India's pulp, paper, and paper board production was 10.29 million metric tons in 2010 (Kulkarni 2013). However, the paper industries in India are not as modernized as in western countries, with more than 55% of these mills lack adequate effluent treatment facilities. Thus, paper mill effluents (PME) are released into the environment without any prior treatments (Devi et al. 2011, Mishra et al. 2013). Most of these mills often discharge effluents in and around water bodies and cause aquatic pollution (Iqbal et al. 2013). PME are also one of the most significant source of aquatic pollution worldwide as they contain unknown chemical mixtures, at times containing hundreds of compounds, that often affect aquatic organisms (Pacheco and Santos 2002). PME have often been reported to cause steady declines in aquatic fauna, particularly fishes (Sepúlveda et al. 2003). Several short- and long-term studies conducted either in the field or laboratory have assessed several endpoints. When tested in high concentrations, PME affect sexual differentiation in fish (Afonso et al. 2002), reduce sex steroid hormone levels (Gagnon et al. 1994), gonad size, and fecundity, delay sexual maturity, and even cause male-biased sex ratios among fish embryos

(Parrott and Blunt 2005). Additionally, the effects of PME on physiology and biochemistry (Jeney et al. 1996), stress response (Afonso et al. 2003), genotoxicity (Pacheco and Santos 1997, 1999), and increased hepatic microsomal DNA adducts (Wilson et al. 2001) have been documented. Work showing that histopathology is a sensitive indicator with which to evaluate the toxicity of PME is scarce. As an indicator of exposure to contaminants, histology is a useful tool for assessing the direct toxic effects of chemical compounds on target organs of fish in laboratory experiments (Costa et al. 2011). Additionally, histopathological investigations have the capacity to differentiate between organ lesions induced by diseases from those caused by exposure to pollutants.

Cachar Paper Mill (CPM) in Assam is a bleach kraft mill situated by the confluence of Dhaleswari and Barak rivers (24°51'16.9"N 92°35'33.6"E). The waters of these two rivers are used by the local people for drinking and domestic needs and irrigation. The mill empties its effluents into both of these rivers and at the adjoining agricultural fields. Consequently, there is increasing public concern regarding the health hazards and ecological risks associated with effluents from this mill. The present study first analyzed the physicochemical parameters of the effluent sample from CPM using different multivariate statistical techniques, such as cluster analysis (CA), the Pearson correlation coefficient, and principal component analysis (PCA), which helped to interpret the complex mixtures of chemicals present in the effluents and to better understand the water quality. These techniques also helped identify possible factors and/or sources that influenced the water systems, and they also served as invaluable tools for environmental monitoring and risk assessment (Lee et al. 2001, Wunderlin et al. 2001, Reghunath et al. 2002, Simeonov et al. 2004, Devi et al. 2011). The study then included an integrated approach to assessing the short- and long-term responses of fingerling *Cyprinus carpio* L. to mill effluents. Short-term responses included mortality tests and the behavior study, for exposure periods of 24, 48, 72, and 96 h, while the long-term response included histopathological changes in gills and livers over the course of 7, 14, 21, and 28 d. Common carp, *C. carpio,* fingerlings were chosen since larval mortality was naturally high in most instances, and it was especially aggravated by environmental contaminants. Therefore, toxicological assessment of developing fish stages had a high predictive value in the risk assessment. Carp fingerlings also had the favorable experimental properties; they were of a small size, and they were easy to collect and maintain under laboratory conditions. They were also chosen because of their widespread culture in various fisheries in this region. Additionally, long-term responses could be very useful in assessing the chronic effects of contaminants at low doses, which will be very effective in predicting natural situations.

Material and methods

Study site, collection of effluent samples, and physicochemical analysis

CPM is a bleach kraft mill that mostly uses bamboo plants as the pulp source. The production of this paper mill for 2008-2009 was 65,012 tons (http://www.hindpaper.in/mills/cachar.htm). The mill uses both primary treatment, which includes the chemo-mechanical treatment of effluents, and secondary treatment, that includes biological treatments. The pulp process of the mill includes cooking, washing, bleaching (calcium hypochloride, H_2O_2, chlorine, and chlorine dioxide). The mill has several outlets, including the one indicated in Fig 1, which discharges near both rivers. This outlet is approximately 1.1 km from the mill, to which it is connected by an underground drainage pipe.

Samples were collected in triplicate with clean 1 L water sampling cans. The samples were transported to the laboratory for physicochemical analysis and stored for further use. The pH and conductivity was measured with a Metller Toledo pH meter and a Systronic conductivity meter. Dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS),

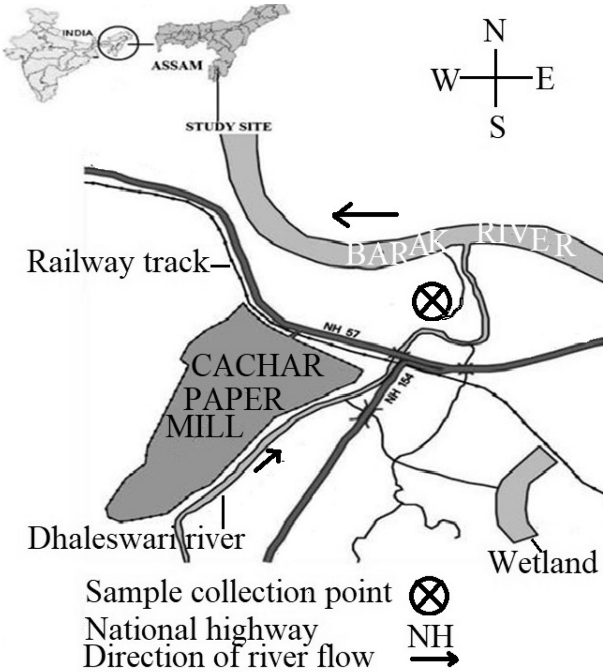

Figure 1. Map of sample collection site.

total alkalinity (TA), total hardness (TH), free chlorine (Cl), chloride (Cl^-), sulfite, and nitrite were estimated in accordance with standard methods (APHA 2005). Water samples for heavy metal analyses were filtered and digested with 10 ml of concentrated analytical grade nitric acid to samples of 250 ml. The solutions were evaporated in a crucible to approximately 5 ml, then filtered into 20 ml standard flasks, and made up to the mark with distilled water. The water extract was analyzed for metals/metalloids such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), and zinc (Zn) in a Vario 6 model graphite furnace atomic absorption spectrometry (GF-AAS) by Analytik Jena. For quality control and quality assurance, standard metal reference material (Merck, Germany) was used for calibration and quality assurance for each analytical batch. The analytical data quality of the metals were ensured by repeated analyses of quality control samples (n = 3) and by results that were within certified values. The detection limits for As, Cd, Cr, Cu, Fe, Pb, and Zn, were 0.4, 0.007, 0.1, 0.19, 0.1, 0.08, and 0.002 $\mu g\ L^{-1}$, respectively.

Multivariate statistical methods for effluent sample analysis

The multivariate analysis of the effluent data sets was performed with CA, Pearson correlation coefficient, and PCA. CA and PCA were standardized through z-scale transformations because of the wide differences in data dimensionality (Liu et al. 2003, Simeonov et al. 2003). The hierarchical agglomerative clustering approach provided intuitive similarity relationships between any one sample and the entire data set and was typically illustrated by a dendrogram (McKenna 2003). The Euclidean distance usually gave the similarity between two samples and distances could be represented by the difference between the analytical values of the samples (Otto 1998). Liu et al. (2003) classified factor loadings as strong if the corresponding loading values were > 0.75 and moderate, if they were < 0.75-0.5 >. PCA was designed to transform the original variables into new, uncorrected variables, referred to as principal components, which were linear combinations of the original variables. Factor analysis was performed on the principal components. Eigenvalues > 1.0 were considered significant (Ouyang et al. 2006) and an equal number of varimax factors was obtained for the effluent samples through factor analysis performed on the principal component. All statistics were processed with SPSS 19 statistical software for Windows.

Fish maintenance

C. carpio fingerlings of similar lengths (4.5 ± 0.1 cm) and weights (3.3 ± 1.0 g) were collected from a local fishery facility with no pollution load (preliminary tests for the presence of the common elements of As, Cd, Cr, Cu, Pb, Zn, and Se were negative). The fish were acclimatized under laboratory conditions seven days prior to the experiment in 20 L glass tanks, and they were fed commercial fish feed twice daily. Acclimatization was conducted in a temperature controlled room at 25°C, with a light-dark cycle of 12:12 h. Chlorine-free tap water was used throughout the experiments, and it had a pH of 6.4-6.7, a total hardness (as $CaCO_3$) of 225-282 mg L^{-1}, and nitrite (as $NaNO_2$) of 385-410 mg L^{-1} (tested with respective test kits procured from Himedia). DO was estimated to be 5.5 ± 0.24 mg L^{-1}.

Acute toxicity study – LC_{50} and behavior study

The effluent collected from the discharge site was considered to be 100% and served as the stock solution. Serial dilutions of the stock solution were prepared using previously aerated, stored tap water. Static renewal acute toxicity tests were conducted with ten fish in each graded concentration. The fish were placed in glass aquaria containing dechlorinated tap water. PME was added in concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% (v/v). Fish kept in chlorine free tap water served as the control. The fish were not fed 24 h prior to the acute toxicity tests. The test solution was replaced and mortality monitored at 24, 48, 72, and 96 h. Dead fish were removed as the tests proceeded. The number of dead fish per group was recorded against the time of their death in tabular form. The entire experiment was repeated thrice. The 24, 48, 72, and 96 h LC_{50} values of PME were calculated with the Probit method (Finney 1971) and the relation between acute dose and response (mortality) was computed with the Pearson correlation coefficient (r) using SPSS statistical software for Windows. The behavioral patterns of the fish were monitored regularly under 100% (v/v) PME treatments. The mean prevalence of each behavior parameter was categorized as none (–), mild (+, < 25% of response), moderate (+ +, 25-50% of response) and severe (+++, > 50% of response) (Shah 2002).

Chronic Toxicity Study on Gill and Liver Histopathology

The sub-lethal test concentration of 2.9% (v/v) PME (1/5th of 96-h LC_50 value) was selected for the chronic studies. Five fish were kept in each test concentration for histopathological examinations. Gill and liver tissues were removed after treatment periods of 28 d. The fish were anesthetized and prepared for histopathological analysis. Small pieces of organs from controls and treated fish were fixed in 10% formalin fluid for 24 h then washed with tap water. They were dehydrated through a graded series of ethanol, cleared in xylene, infiltrated in paraffin, and sectioned at a thickness of 5 μm. The sections were stained with hematoxylin and eosin, examined by light microscopy, and then photographed (Olympus Microscope, model CX41RF with Olympus digital Camera, model: E-420). The histopathological lesions in the tissues were examined in randomly selected five sections from 5 fish of each group. The mean prevalence of each histopathological parameters was categorized as none (–), mild (+, < 25% of sections), moderate (+ +, 25-50% of sections) and severe (+ + +, > 50% of sections) (Mishra and Mohanty 2008).

Results and Discussion

Effluent Quality Analysis

The physicochemical parameters of the effluent samples are presented in Table 1. The sample was reddish brown in color which might have been due to the lignin from the pulping of bamboo shoots, the chief source raw material used for paper production in this mill. This dark color of the effluent could limit feeding efficiency in aquatic organisms (van den Heuvel et al. 2008). The sample had a foul smell and a high TDS concentration, which could be injurious to fish. In the present analysis, samples had 600 mg L^{-1} chloride from the use of chlorine in the bleaching process. Chloride is highly soluble in water and cannot be eliminated by biological or chemical treatments. The pH of the effluent was 7.3 ± 0.1. The effluent quality was compared with the maximum tolerance limits for discharged effluents in surface waters set by both Environmental Protection Act (2002) and Indian Standards (1981) and for irrigation purposes (Environment Studies Board 1973), but not for drinking water (Indian Standards 2004). The results

Table 1
Physicochemical characteristics of paper mill effluent sample collected from an effluent discharge site

Parameters	Effluent sample	Maximum tolerance limits for industrial effluents discharged (1981)[a]		Drinking water standards (2004)[b]	Effluent discharge in water (2002)[c]	Irrigation standards (1973)[d]
		Surface water	Agriculture			
Colour	Reddish brown	NA	NA	NA	Agreeable	NA
Odor	Pungent	NA	NA	NA	Agreeable	NA
Conductivity (μS cm^{-1})	279.3 ± 7.2	NA	NA	NA	NA	NA
Dissolved Oxygen (mg L^{-1})	3.23 ± 0.180	NA	NA	NA	NA	NA
TDS (mg L^{-1})	382.6 ± 17.7	2100	2100	NA	2000.0	NA
pH	7.33 ± 0.1	5.5-9.0	5.5-9.0	09-maj	6.5-8.5	NA
Total alkalinity (mg L^{-1})	640.3 ± 6.5	NA	NA	NA	600.0	NA
Total hardness (mg $CaCO_3$ L^{-1})	460.7 ± 4.86	NA	NA	NA	600.0	NA
Free chlorine (mg L^{-1})	Absent	1.0	NA	0.5	0.2	NA
Chloride (mg L^{-1})	586.6 ± 13.2	750	NA	750	1000	NA
Sulfite (mg L^{-1})	Absent	NA	NA	NA	NA	NA
Nitrite (mg L^{-1})	5129.4 ± 111.9	1	NA	1	NA	NA
BOD (mg L^{-1})	200.6 ± 7.81	30	100	40	NA	NA
COD (mg L^{-1})	1675.3 ± 69.6	250	NA	120	NA	NA
Cu (mg L^{-1})	4.06 ± 0.06	3.0	NA	0.5	1.5	0.2
Cd (mg L^{-1})	0.723 ± 0.02	2.0	NA	0.01	0.001	0.01
Cr (mg L^{-1})	34.23 ± 0.35	0.1	NA	0.05	0.05	0.1
Fe (mg L^{-1})	476.0 ± 0.39	2.0	5	2.0	1.0	5
Zn (mg L^{-1})	29.5 ± 0.13	5.0	NA	0.1	15	2
Pb (mg L^{-1})	3.05 ± 0.02	0.1	NA	0.05	0.05	5
As (mg L^{-1})	3.13 ± 0.105	0.2	0.2	0.1	0.05	0.1

[a]Indian standards (1981), [b]Indian standards (2004), [c]Environmental Protection Act (2002), [d]Environment Studies Board (1973), NA – guideline value not available

revealed that almost all the parameters, including the heavy metal/metalloid contents, exceeded permissible limits.

In this study, hierarchical agglomerative CA yielded a dendrogram (Fig. 2) that groups all 17 physicochemical parameters present in the PME into two statistically significant clusters at $(D_{link} / D_{max}) \times 100 < 60$. The primary objective of this technique was to assemble parameters based on the associations between them (Shrestha and Kazama 2007). The results indicated that the CA technique was useful in offering a reliable classification of the physicochemical parameters present in the mill effluent that provides valuable information regarding the mode of action of PME and its probable toxicities. Other reports indicate the successful use of this technique in Indian water quality

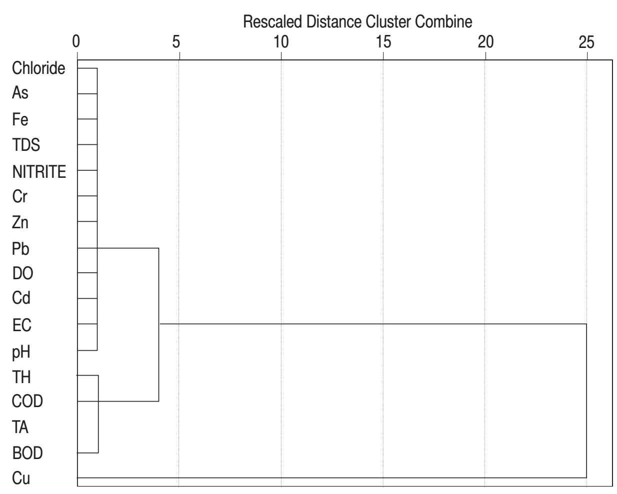

Figure 2. Dendrogram showing the clustering of various physicochemical parameters of paper mill effluents using average linkage (among groups).

Table 2
Pearson correlation matrix between different physicochemical parameters in effluent (n = 9)

	Conductivity	DO	TDS	pH	TH	TA	Chloride	Nitrite	BOD	COD	Cu	Cd	Cr	Fe	Zn	Pb	As
DO	1.0**	-															
TDS	0.99**	0.99**	-														
pH	0.99**	0.99**	0.99**	-													
TH	0.92**	0.92**	0.94**	0.91**	-												
TA	0.94**	0.94**	0.96**	0.93**	0.99**	-											
Chloride	0.99**	0.99**	0.99**	0.99**	0.95**	0.97**	-										
Nitrite	0.99**	0.99**	0.99**	0.98**	0.96**	0.98**	0.99**	-									
BOD	1.0**	0.94**	0.95**	0.92**	0.99**	1.0**	0.96**	0.97**	-								
COD	0.92**	0.92**	0.94**	0.91**	1.0**	0.99**	0.95**	0.96**	0.99**	-							
Cu	0.66	0.66	0.62	0.68*	0.32	0.39	0.59**	0.561	0.368	0.33	-						
Cd	1.0**	1.0**	0.99**	0.99**	0.92**	0.94**	0.99**	0.99**	0.94**	0.92**	0.66	-					
Cr	0.99**	0.99**	0.99**	0.98**	0.96**	0.98**	0.99**	1.0**	0.97**	0.97**	0.55	0.99**	-				
Fe	0.99**	0.99**	1.0**	0.99**	0.95**	0.97**	1.0**	0.99**	0.96**	0.95**	0.60	0.99**	0.99**	-			
Zn	0.99**	0.99**	0.99**	0.98**	0.96**	0.97**	1.0**	1.0**	0.97**	0.96**	0.57	0.99**	1.0**	0.99**	-		
Pb	0.98**	0.98**	0.99**	0.98**	0.97**	0.98**	0.99**	0.99**	0.98**	0.97**	0.52	0.98**	1.0**	0.99**	0.99**	-	
As	0.99**	0.99**	0.99**	0.99**	0.95**	0.97**	1.0**	0.99**	0.96**	0.95**	0.59	0.99**	0.99**	1.0**	1.0**	0.99**	-

*Significant correlation at the level P < 0.05, **Significant correlation at the level P < 0.01

monitoring (Singh et al. 2004, Singh et al. 2005, Parashar et al. 2008, Devi et al. 2011).

Correlation coefficients among different physicochemical parameters in effluents are presented in Table 2. Except for Cu, positive correlations ($P < 0.01$) were found among different parameters such as DO, TDS, conductivity, total hardness, total alkalinity, nitrite, BOD, COD, and heavy metals. Cu was positively correlated only with pH ($P < 0.05$) and chloride ($P < 0.01$). Factor analysis was performed on the principal components and the corresponding variable loadings, and the explained variance is presented in Table 3. For effluent samples, only one PC explained 94.2% of the total variance, and, except Cu, all 16 physicochemical parameters formed a part of a highly correlated cluster with high factor loadings of > 0.9. PCA analysis and the correlation matrix (CM) have also been used as effective tools in the source identification of heavy metals in other studies (Han et al. 2006, Mico et al. 2006).

Acute toxicity-LC_{50} and behavior study

The fish held under control conditions were healthy and normal and no mortality was recorded. The median lethal concentrations (LC_{50}) of paper mill effluent at 24, 48, 72, and 96 h were 42, 25, 18.6, and 14.5% (v/v), respectively (Table 4). During acute toxicity studies, the exposed fish showed erratic swimming patterns, sudden spurts of fast swimming, gulping air followed by lethargy, and finally death (Table 5). In another study, 96 h LC_{50} for *Puntius sophore* (Hamilton) was estimated to be 1.5% and 16.5% in aerated and non-aerated groups, respectively (Rajendra et al. (1991). The LC_{50} values also varied with the species tested. Pathan et al. (2009) found 96-h LC_{50} to be 9.5% in *Rasbora daniconius* (Hamilton), while Wahbi et al. (2004) found 96-h LC_{50} to be 3.5% in *Lithognathus mormyrus* (L.). The results of the present study showed that carp fingerlings were sensitive to PME.

The fish in the current study exhibited abnormal swimming and disturbed orientation. These behavioral anomalies in orientation and locomotion can be related to the impairment of sensory organ systems particularly the mechano- and chemo-receptor systems (Gardner 1975). The opercular movements of the dying fish became extremely slow, and they were observed to secrete thick coats of mucus. Clotfelter et al. (2006) observed aggressive behavior, increased surface breathing, and opercular movements in

Table 3
Total variance and component matrix (one principal components selected) for effluent physicochemical parameters (n = 9)

	Eigenvalues	% of variance	Cumulative %	Parameters	PC1
1	16.01	94.22	94.22	pH	0.990
2	0.982	5.78	100.0	Conductivity	0.994
3	7.25E-16	1.80	100.0	DO	0.993
4	4.8E-16	0.00	100.0	TDS	0.998
5	4.14E-16	0.00	100.0	TA	0.978
6	3.29E-16	0.00	100.0	TH	0.960
7	2.64E-16	0.00	100.0	Chloride	0.999
8	2.46E-17	0.00	100.0	Nitrite	0.999
9	6.84E-17	0.00	100.0	BOD	0.972
10	4.36E-17	0.00	100.0	COD	0.963
11	8.97E-17	0.00	100.0	Cu	0.575
12	-7.6E-17	0.00	100.0	Cd	0.993
13	-2.7E-16	0.00	100.0	Cr	0.999
14	-3.9E-16	0.00	100.0	Fe	0.999
15	-3.5E-16	0.00	100.0	Zn	0.999
16	-5.8E-16	0.00	100.0	Pb	0.998
17	-7.1E-16	0.00	100.0	As	0.999

PC1 is the Principal Components 1. Factor loadings > 0.75 is considered as strong (as per Liu et al. 2003)

Table 4
Mortality and LC_{50} values of paper mill effluent on *C. carpio* fingerlings

Paper mill effluent	No. of alive fish				
(%)	24 h	48 h	72 h	96 h	Mortality at 96 h (%)
0	10	10	10	10	0
10	10	8	7	6	40
20	7	6	4	3	70
30	7	5	4	2	80
40	6	4	3	1	90
50	5	4	2	0	100
60	4	2	1	0	100
70	4	1	0	0	100
80	4	0	0	0	100
90	0	0	0	0	100
100	0	0	0	0	100
Pearson ‘r’	0.965	0.971	0.919	0.814	
LC_{50} (95% CI)	42.10 ± 5.21 (31.8-53.9)	25.30 ± 3.53 (18.0-32.5)	18.63 ± 2.88 (12.9, 24.4)	14.53 ± 2.07 (10.2, 18.8)	

LC_{50} values are mean ± SE; Confidence intervals 95% (CI) are indicated in parenthesis

stressed *Mystus vittatus* (Bloch), which would indicate sustained respiratory discomfort in the toxic whole paper mill effluent. Therefore, the behavioral changes, particularly those concerned with respiratory insufficiency, observed in the present study could have contributed to mortality in these stressed fish.

Chronic study with histopathology

Gills

The results of histological examinations of gill tissues exposed for various periods to PME are summarized in Table 6. The gills of *C. carpio* under control conditions at 7, 14, 21, and 28 d exhibited normal gill

Table 5
Impact of paper mill effluent (100%, v/v) on the behavioral pattern of *C. carpio* fingerlings up to 96 h of exposure

Parameters	Control	24 h	48 h	72 h	96 h
Hyperactivity	-	++++	+++	++	+
Loss of balance	-	+	++	+++	++++
Gulping of air	-	++++	+++	++	+
Rate of swimming	-	++++	+++	++	+
Rate of opercular activity	-	++++	+++	++	+
Mucous filled gills	-	+	++	+++	++++

- indicates normal behaviour,
+ indicates increase or decrease in the level of behavioural parameters

Table 6
Semiquantitative analysis of gill leisons from paper mill effluent treatment group (2.9 %, v/v) of *C. carpio* fingerlings at various exposure durations

Gill Lesion	7 d	14 d	21 d	28 d
Mucous secretion	+	++	++	+++
Mucous cell proliferation	-	++	+++	+++
Sloughing of epithelium	+	+	++	+++
Leucocyte infiltration	+	+	++	+++
Curling of tip of SL	+	++	+++	+++
Clubbing of SL	+	++	+++	++
Aneurism	-	-	-	+++
Fusion of SL	-	-	-	++
Damaged of SL	-	-	-	+++
LBS dilation	+	+	++	++
Hypertrophy	+	+	++	++
Hyperplasia	-	+	++	++
haemmorhage of PL	-	+	++	++
lifting of epithelium	-	-	+	++
LBS constriction	-	-	+	+
LBS conjestion	-	-	++	+++

- none, + mild, ++ moderate, +++ severe occurrence, SL – secondary lamellae, PL – primary lamellae; LBS – lamellar blood sinus

architecture with primary lamellae (PL), secondary lamellae (SL), lamellar blood sinus (lbs), and pillar and epithelial cells (Fig. 3a). However, after 7 d of exposure, in comparison to the control, the gills exhibited mucous secretion, sloughing of the epithelium, leucocyte infiltration, curling of the tip of the SL, dilation of lbs, and hypertrophy and clubbing of the SL (Fig. 3b). After 14 d of exposure, the gills showed mucous cell proliferation, sloughing of the epithelium, curling of the SL, hypertrophy, hyperplasia, clubbing of the SL, and PL hemorrhaging (Fig. 3c). However, after 21 d of exposure, gills exhibited PL hemorrhaging, lifting of the epithelium, mucous cell proliferation, leucocyte infiltration, hyperplasia, V-shaped curl of the SL, and the constriction and congestion of lbs (Fig. 3d). After 28 d of exposure, the gills exhibited PL hemorrhaging, mucous cell proliferation, fusion of the SL, aneurism, damaged SL, leucocyte infiltration, and hypertrophy of epithelium (Fig. 3e). The responses became more severe as exposure continued.

Fish gills have a large surface area through which gaseous exchange between the fish and the external medium occurs, and they also perform the functions

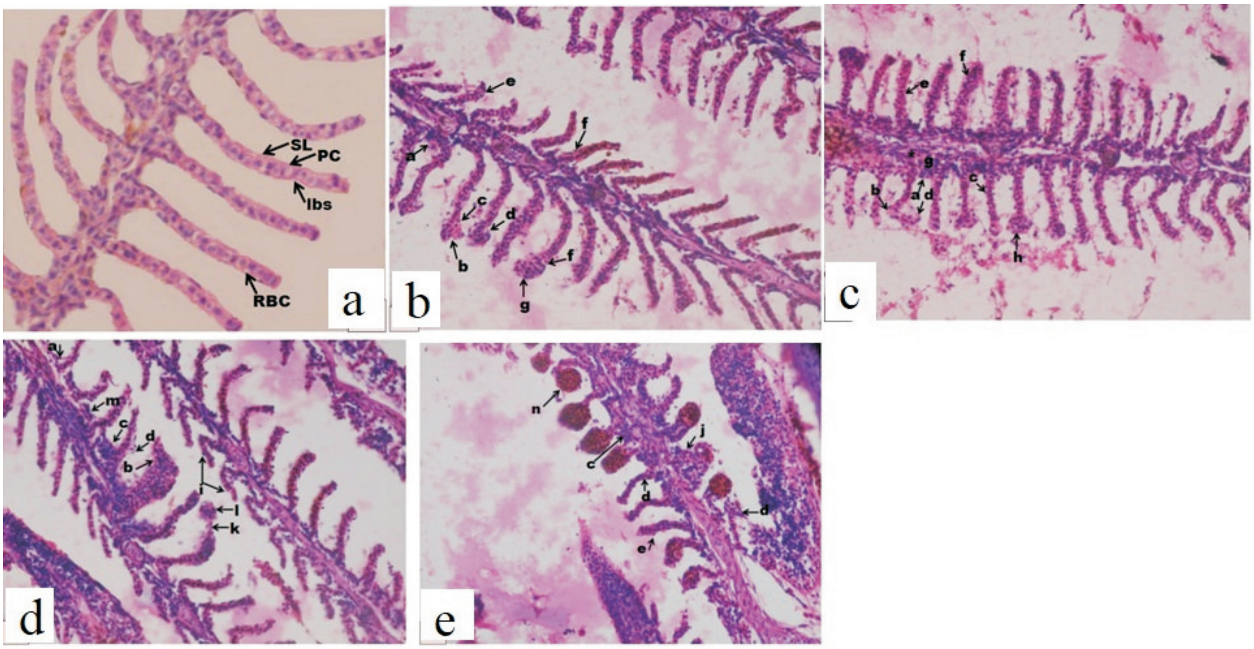

Figure 3. Gill of *Cyprinus carpio*. a) Gill of control *C. carpio* exhibiting primary lamellae (PL), secondary lamellae (SL), lamellar blood sinus (lbs) with RBC, pillar cell (pc). b) Gill after 7 d exposure to 2.9% PME exhibiting mucous secretion (a), sloughing of the epithelium (b), leucocyte infiltration (c), curling of the tip of the SL (d), dilation of lbs (e), hypertrophy (f) and clubbing of SL (g), (× 400). c) Gill after 14 d exposure exhibiting mucous cell proliferation(a), sloughing of the epithelium (b), leucocyte infiltration (c), curling of the SL (d), hypertrophy (e), hyperplasia (f), hemorrhage of the PL (g), clubbing of the SL (h), (× 400). d). Gill after 21 d exposure exhibiting hemorrhage of the PL (a), lifting of the epithelium (b), mucous cell proliferation(c), leucocyte infiltration (d), hyperplasia (e), "V" shaped curl of the SL (f), lbs constriction (g), congestion of lbs (h), (× 400). e) Gill after 28 d exposure exhibiting hemorrhage of the PL (a), mucous cell proliferation(b), fusion of the SL (c), aneurism (d), damaged SL (e), leucocyte infiltration (f), and hypertrophy of the epithelium (g) (× 400).

of osmoregulation and excretion (Chezhian et al. 2009). However, changes in gills can become progressively worse when fish are exposed persistently to contaminants, and eventually they compromise organ function. Hence, gill histopathological changes can be used as biomarkers in fish exposed to contaminants (Garmendia et al. 2010). In the present study, common carp gills were monitored weekly and several anomalies were observed. However, the severity of effects increased progressively and peaked at 28 d. Gill hyperplasia, lamellar fusion, and mucus secretion serve as defense responses of the fish to effluents (Das and Gupta 2012). Mucous is essential for fish respiration and osmoregulation and also plays a protective role since it appears soon after effluent exposure and prevents toxins from entering the gills. On the other hand, the proliferation of inter-lamellar cells and the consequent fusion of adjacent secondary lamellae that formed club-shaped lamellae and caused aneurisms were some of the anomalies observed only after 28 d of exposure. Similar observations were also made in European eel after 30 d in 12.5% PME treatments (Pacheco and Santos 2002). Lamellar aneurism is characterized by blood extravasations inside the lamellae and the rupture of the pillar cell system, with the consequent blood vessel dilation (Heath 1995). Similar incidences of damaged gills were observed in rainbow trout (Couillard et al. 1988) and perch (Lindesjöö and Thulin 1994) that were exposed to PME. Lamellar fusion is also reported in fish exposed to effluents from a bleached paper mill (Pacheco and Santos 2002). Vascular congestion, which was observed in the current study, also impairs blood flow and compromises the basic gill function of gas exchange (Heath 1995). Apart from impairing this important function, congestion also interferes with other functions such as the maintenance of acid-base and osmotic balances (Garcia-Santos et al. 2007), which, however, was not studied in the present investigation. Overall, the alterations in gill histology in response to PME represent adaptive strategies for the conservation of some physiological functions in the face of a stressor. These histopathological alterations may have important adverse consequences for fish health, particularly from the obstruction of oxygen diffusion across the gills and the impairment of osmoregulatory function. Several other studies report similar observations, including information regarding the gills of Nile Tilapia, *Oreochromis niloticus* (L.), exposed to treated sewage water (Fontaínhas-Fernandes et al. 2008) or cassava effluents (Wade et al. 2002), of cunner, *Tautogolabrus adspersus* (Walbaum), exposed to municipal and industrial effluents (Billiard and Khan 2003), and of certain neotropical fish exposed to chemical factory effluents (Camargo and Martinez 2007).

Liver

The results of histological examinations of liver tissues exposed for various periods to PME are summarized in Table 7. The livers of control *C. carpio* exhibited normal architecture with hepatocyte with nuclei, central veins (CV), sinusoids, and pancreatic areas (Fig. 4a). The livers of *C. carpio* after 7 d of exposure exhibited inflammatory infiltration, cytoplasmic inclusion bodies (CIB), vacuolar degeneration, and fatty degeneration (Fig. 4b). After 14 d of exposure, the livers exhibited cytoplasmic inclusion bodies, vacuolar degeneration, pyknosis, necrotic areas, blood filled central veins, CV compartmentation, and sinusoidal congestion (Fig. 4c). After 21 d of exposure, the livers exhibited cytoplasmic inclusion bodies, fatty degeneration, necrosis, karyolysis, inflammatory infiltration, sinusoidal congestion and dilation, blood filled central veins, CV compartmentation, vacuolar degeneration, and distorted pancreatic areas (Fig. 4d). After 28 d of exposure, the livers exhibited hepatocyte vacuolation, piecemeal necrosis, vacuolar degeneration, and karyolysis along with sinusoidal congestion and dilatation, and inflammatory infiltration in the pancreatic areas (Fig. 4e).

The liver plays an important role in vital functions, basic metabolism, and the accumulation, transformation, and excretion of contaminants (Figueiredo-Fernandes et al. 2006). Inflammatory cell infiltrates, detected in the sinusoids of carp liver might be a generalized reaction to contaminant exposure. Such infiltrates often caused sinusoidal congestion. Necrosis indicated the degeneration of structural proteins in the hepatocyte membrane, and pyknosis signified altered protein synthesis. Previously, several investigators linked these cellular deteriorations to PME exposure (Santos et al. 1990, Axelsson and Norrgren 1991, Pacheco and Santos

Table 7
Semiquantitative analysis of liver leisons from paper mill effluent treatment group (2.9 %, v/v) of *C. carpio* fingerlings at various exposure durations

Liver Lesion	7 d	14 d	21 d	28 d
Inflammatory infiltration	+	++	+++	+++
Vacoular degeneration	+	+	++	+++
Fatty degeneration	+	++	++	+++
Cytoplasmic inclusion bodies	+	++	+++	-
Pycnotic nucleus	-	+	+	+
Necrotic areas	-	+	++	+++
Karyolysis	-	-	+	+++
Compartmentation of central vein	-	+	++	+++
Blood filled central vein	+	+	++	+++
Conjestion of sinusoid	+	+	++	+++
Dilated sinusoid	-	+	++	+++
Distorted pancreatic area	-	-	+	+

- none, + mild, ++ moderate, +++ severe occurence

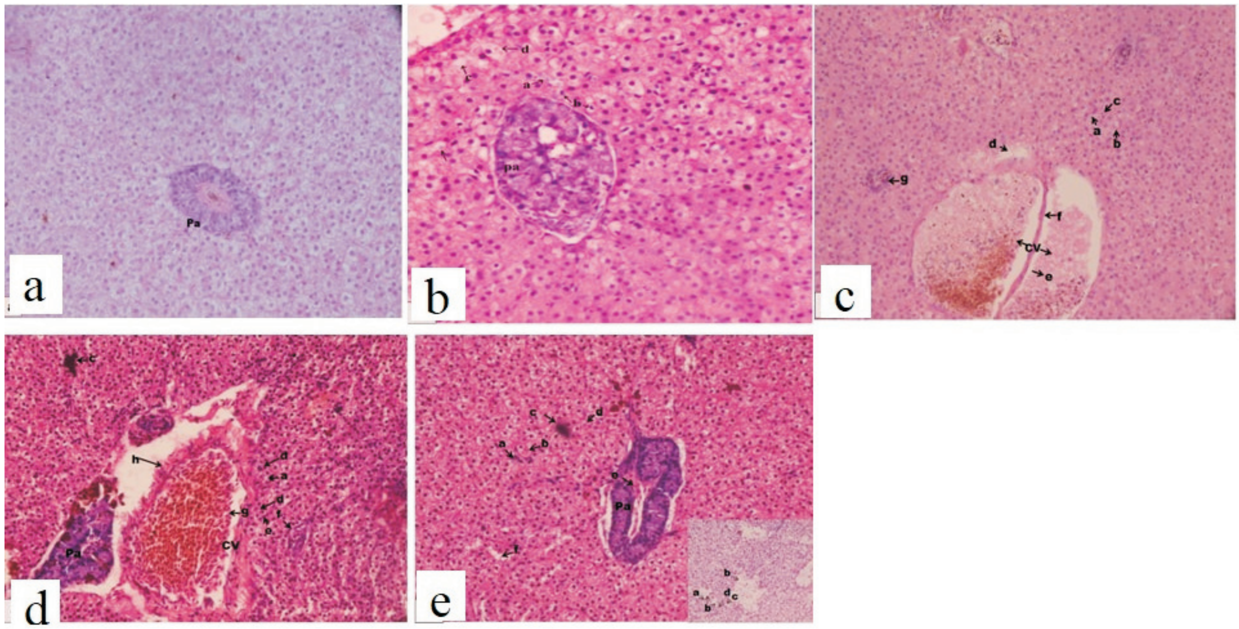

Figure 4. Liver of *Cyprinus carpio*. a) Liver of control *C. carpio* exhibiting pancreatic areas (pa) (× 400). b) Liver 7 d after exposure to 2.9% PME exhibiting inflammatory infiltration (a), cytoplasmic inclusion bodies (b), vacuolar degeneration (c), fatty degeneration (d), (× 400). c) Liver 14 d after exposure exhibiting cytoplasmic inclusion bodies (a), vacuolar degeneration (b), pyknosis (c), necrotic areas (d), blood filled central vein (e), CV compartmentation (f), sinusoidal congestion (g) (× 400). d) Liver 21 d after exposure exhibiting cytoplasmic inclusion bodies (a), fatty degeneration (b), necrosis (c), karyolysis (d), inflammatory infiltration (e), sinusoidal congestion and dilation (f), blood filled central vein (g), CV compartmentation (f), and distorted pancreatic area (pa) (× 400). e) Liver 28 d after exposure exhibiting hepatocyte vacuolation (a), piecemeal necrosis (b), vacuolar degeneration (c), karyolysis (d), inflammatory infiltration (e) in the pancreatic area (pa) (× 400).

2002). In the current study, single cell necrosis that was observed at the shorter exposures later occurred along widespread necrotic areas, which imparted a piecemeal appearance to the tissues. Liver vacuolization was also detected in this study, and similar anomalies were also observed in PME-treated fish (Axelsson and Norrgren 1991). Winter flounder, *Pleuronectes americanus* (Walbaum) exposed to PME had focal vacuolation and enlarged livers (Khan et al. 1994). Similar hepatic damage was also observed in gudgeon, *Gobio gobio* (L.) and mullet, *Mugil cephalus* L. exposed to contaminants from a wastewater treatment plant (Pinto et al. 2010). The high incidence of CIB might be correlated with increased metal exposure in tissues (Peplow and Edmonds 2005) and the metallothionein-based hepatic sequestration of tissue metals (Greenfield et al. 2008). Similar CIBs were also observed in the livers of splittail fish, *Pogonichthys macrolepidotus* (Ayres), and were highly correlated with contaminants containing Ag, Cu, Mo, and Zn (Greenfield et al. 2008). The chlorine in the effluents might also contribute to histopathological alterations.

Thus, from the present study, it is concluded that the paper mill effluent was extremely toxic to fish even in very low concentrations. Such contaminants weaken fish rendering them more susceptible to mortality from other causes. Although the mill performs both primary and secondary treatments before discharging effluents, these processes are inadequate. Therefore, better treatment of effluents prior to their release into the environment is recommended.

Acknowledgement. We would like to thank Biotech Hub, Life Science, Assam University, Silchar for providing microscopic facilities.

Author contributions. S.D. had performed all the experiments as a part of her doctoral research work; M.D.C. was also involved in partially conceptualizing the work and provided instrumentation facilities; S.D. has conceptualized and designed the work, as well as written the manuscript.

References

Afonso L.O.B., Basu N., Nakano K., Devlin R.H., Iwama G.K. 2003 – Sex-related differences in the organismal and cellular stress response in juvenile salmon exposed to treated bleached kraft mill effluent – Fish Physiol. Biochem. 29: 173-179.

Afonso L.O.B., Smith J.L., Ikonomou M.G., Devlin R.H. 2002 – Y-Chromosomal DNA markers for discrimination of chemical substance and effluent effects on sexual differentiation in salmon – Environ. Health Persp. 110: 881-887.

APHA 2005 – Standard methods for the examination of water and wastewater, 21st ed. – Washington DC, USA.

Axelsson B., Norrgren L. 1991 – Parasite frequency and liver anomalies in tree-spined stickleback, *Gasterosteus aculeatus* (L.), after long-term exposure to pulp mill effluents in marine mesocosms – Arch. Environ. Contam. Toxicol. 21: 505-513.

Billiard S.M., Khan R.A. 2003 – Chronic stress in cunner, *Tautogolabrus adspersus*, exposed to municipal and industrial effluents – Ecotox. Environ. Safe. 55: 9-18.

Camargo M.M., Martinez C.B. 2007 – Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream – Neotrop. Ichthyol. 5: 327-336.

Chezhian A., Kabilan N., Kumar T.S. 2009 – Impact of chemical factory effluent on the structural changes in gills of fresh water fish *Cyprinus carpio* var, *communis* (Linnaeus, 1758) – J. Basic Appl. Biol. 3: 28-35.

Clotfelter D., Ethane C., Alison R. 2006 – Behavioural changes in fish exposed to phytoestrogens – Environ. Pollut. 144: 833-839.

Costa P.M., Caeiro S., Lobo J., Martins M., Ferreira A.M., Caetano M., Vale C., DelValls T.A., Costa M.H. 2011 – Estuarine ecological risk based on hepatic histopathological indices from laboratory and in situ tested fish – Mar. Pollut. Bull. 62: 55-65.

Couillard C.M., Berman R.A., Panisset J.C. 1988 – Histopathology of rainbow trout exposed to a bleached kraft pulp paper mill effluent – Arch. Environ. Contam. Toxicol. 17: 319-323.

Das S., Gupta A. 2012 – Effects of cadmium chloride on oxygen consumption and gill morphology of Indian flying barb, *Esomus danricus* – J. Environ. Biol. 33: 1057-1061.

Devi N.L., Yadav I.C., Shihua Q.I, Singh S., Belagali S.L. 2011 – Physicochemical characteristics of paper industry effluents - a case study of South India Paper Mill (SIPM) – Environ. Monit. Assess. 177: 23-33.

Environmental Protection Act 2002 – Standards for effluent discharge regulations. General Notice No. 44 of 2003 – Available from http://faolex.fao.org/docs/texts/mat 52519. doc.

Environmental Studies Board 1973 – Trace elements tolerance for irrigation waters – Environment Studies Board, India.

Figueiredo-Fernandes A., Fontaínhas-Fernandes A., Monteiro R.A.F., Reis-Henriques M.A., Rocha, E. 2006 – Effects of the fungicide mancozeb in the liver structure of Nile tilapia, *Oreochromis niloticus* - assessment and quantification of induced cytological changes using qualitative histopathology and the stereological point-sampled intercept method – Bull. Environ. Contam. Tox. 76: 249-255.

Finney D.J. 1971 – Probit analysis – Cambridge University Press, London.

Fontaínhas-Fernandes A., Luzio A., Garcia-Santos S., Carrola J., Monteiro S. 2008 – Gill histopathological alterations in Nile Tilapia, *Oreochromis niloticus* exposed to treated sewage water – Braz. Arch. Biol. Techn. 51: 1057-1063.

Gagnon, M.M., Dodson J.J., Hodson P.V., Van Der Kraak G., Carey J.H. 1994 – Seasonal effects of bleach kraft mill effluent on reproductive parameters of white sucker (*Catostomus commersoni*) populations of the St. Maurice River, Quebec Canada – Can. J. Fish. Aquat. Sci. 51: 337-347.

Garcia-Santos S., Monteiro M., Carrola J., Fontaínhas-Fernandes A. 2007 – Histopathological lesions of tilapia *Oreochromis niloticus* gills caused by cadmium – Braz. J. Vet. Anim. Sci. 59: 376-381.

Gardner G.R. 1975 – Chemically induced lesions in estuarine or marine teleosts – In: The Pathology of fishes (Eds) W.E. Ribelin, G.M. Migaki, Wisconsin Press, Madison: 657-693.

Garmendia L., Soto M., Cajaraville M.P., Marigómez I. 2010 – Seasonality in cell and tissue-level biomarkers in *Mytilus galloprovincialis*: relevance for long-term pollution monitoring – Aquat. Biol. 9: 203-219.

Greenfield B.K., The S.J., Ross J.R.M., Hunt J., Zhang G.H., Davis J.A., Ichikawa G., Crane D., Huang S.S.O., Deng D.F., The F.C., Green P.G. 2008 – Contaminant concentrations and histopathological effects in Sacramento Splittail (*Pogonichthys macrolepidotus*) – Arch. Environ. Contam. Toxicol. 55: 270-281.

Han Y., Du P., Cao J., Posmentier E.S. 2006 – Multivariate analysis of heavy metal contamination in urban dusts of Xi'an, Central China – Sci. Total Environ. 355: 176-186.

Heath A.G. 1995 – Water pollution and fish physiology – CRC, Boca Raton.

Indian standards 1981 – Maximum tolerance limits for industrial effluents discharged – Indian standard (IS 2490: 1981), Part-I, Indian Standards, New Delhi.

Indian standards 2004 – Drinking water specification – Indian standard (IS 10500: 2004), 2nd revision, Indian Standards, New Delhi.

Iqbal S., Younas U., Chan K.W., Saeed Z., Shaheen M.A., Akhtar N., Majeed A. 2013 – Growth and antioxidant response of *Brassica rapa* var. *rapa* L. (turnip) irrigated with different compositions of paper and board mill (PBM) effluent – Chemosphere 91: 1196-1202.

Jeney Z., Valtonen E.T., Jeney G., Jokinen E.I. 1996 – Effects of Pulp and paper mill effluent (BKME) on physiology and biochemistry of the roach (*Rutilus rutilus* L.) – Arch. Environ. Contam. Toxicol. 30: 523-529.

Khan R.A., Barker D.E., Hooper R., Lee E.M., Ryan K., Nag K. 1994 – Histopathology in winter flounder (*Pleuronectes americanus*) living adjacent to a pulp and paper mill – Arch. Environ. Contam. Toxicol. 26: 95-102.

Kulkarni H.D. 2013 – Pulp and Paper Industry Raw Material Scenario - ITC Plantation - a case study – J. Indian Pulp Paper Tech. Assoc. 25: 79-89.

Lee J.Y., Cheon J.Y., Lee K.K., Lee S.Y., Lee M.H. 2001 – Statistical evaluation of geochemical parameter distribution in a ground water system contaminated with petroleum hydrocarbons – J. Environ. Qual. 30: 1548-1563.

Lindesjöö E., Thulin J. 1994 – Histopathology of skin and gills of fish in pulp mill effluents – Dis. Aquat. Organ.18: 81-93.

Liu C.W., Lin K.H., Kuo Y.M. 2003 – Application of factor analysis in the assessment of ground water quality in a Blackfoot disease area in Taiwan – Sci. Total Environ. 313: 77-89.

McKenna Jr. J.E. 2003 – An enhanced cluster analysis program with bootstrap significance testing for ecological community analysis – Environ. Modell. Softw. 18: 205-220.

Mico C., Recatala L., Peris M., Sanchez J. 2006 – Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis – Chemosphere 65: 863-872.

Mishra A.K., Mohanty B. 2008 – Acute toxicity impacts of hexavalent chromium on behavior and histopathology of gill, kidney and liver of the freshwater fish, *Channa punctatus* (Bloch) – Environ. Toxicol. Pharmacol. 26: 136-141.

Mishra S., Mohanty M., Pradhan C., Patra H.K., Das R., Sahoo S. 2013 – Physico-chemical assessment of paper mill effluent and its heavy metal remediation using aquatic macrophytes – a case study at JK Paper mill, Rayagada, India – Environ. Monit. Assess. 185: 4347-4359.

Ouyang Y., Nkedi-Kizza P., Wu Q.T., Shinde D., Huang C.H. 2006 – Assessment of seasonal variations in surface water quality – Water Res. 40: 3800-3810.

Pacheco M., Santos S.A. 1997 – Induction of EROD activity and genotoxic effects by polycyclic aromatic hydrocarbons and resin acids on the juvenile eel (*Anguilla anguilla* L.) – Ecotox. Environ. Safe. 38: 252-259.

Pacheco M., Santos M.A. 1999 – Biochemical and genetic responses of adult eel (*Anguilla anguilla* L.) to resin acids and pulp mill effluent: laboratory and field experiments – Ecotox. Environ. Safe. 42: 81-93.

Pacheco M., Santos M.A. 2002 – Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.) – Ecotox. Environ. Safe. 53: 331-347.

Parrott J.L., Blunt B.R. 2005 – Lifecycle exposure of fathead minnows (*Pimephales promelas*) to an ethinyl estradiol concentration below 1ng/L reduces egg fertilization success and demasculinizes male – Environ. Toxicol. 20: 131-141.

Parashar C., Verma N., Dixit S., Shrivastava R. 2008 – Multivariate analysis of drinking water quality parameters in Bhopal – Environ. Monit. Assess. 140: 119-122.

Pathan T.S., Sonawane D.L., Khillare Y.K. 2009 – Toxicity and behavioural changes in freshwater fish *Rasbora daniconius* exposed to Paper Mill Effluent – Bot. Res. Int. 2: 263-266.

Peplow D., Edmonds R. 2005 – The effects of mine waste contamination at multiple levels of biological organization – Ecol. Eng. 24: 101–119.

Pinto A.L., Varandas S., Coimbra A.M., Carrola J., Fontaínhas-Fernandes A. 2010 – Mullet and gudgeon liver histopathology and macroinvertebrate indexes and metrics upstream and downstream from a wastewater treatment plant (Febros River-Portugal) – Environ. Monit. Assess. 169: 569-585.

Rajendra K.M., Chouhan S., Mishra K.D. 1991 – Toxicity of tannery mill effluent to fish, *Puntius sophor* – J. Tissue Res. 1: 41-48.

Rao S.S., Quinn B.A., Burnison B.K., Hayes M.A., Metcalfe C.D. 1995 – Assessment of the genotoxic potential of pulp mill effluent using bacterial, fish and mammalian assays – Chemosphere 31: 3553-3566.

Reghunath R., Murthy T.R.S., Raghavan B.R. 2002 – The utility of multivariate statistical techniques in hydrogeochemical studies: an example from Karnataka, India – Water Res. 36: 2437-2442.

Santos M.A., Pires F., Hall, A. 1990 – Metabolic effects of kraft mill effluents on the eel *Anguilla anguilla* L – Ecotox. Environ. Safe. 20: 10-19.

Sepúlveda M.S., Quinn B.P., Denslow N.D., Holm S.E., Gross T.S. 2003 – Effects of pulp and paper mill effluents on reproductive success of largemouth bass – Environ. Toxicol. 22: 205-213.

Shah S.L. 2002 – Behavioural abnormalities of *Cyprinion watsoni* on exposure to copper and zinc – Turk. J. Zool. 26: 137-140.

Shrestha S., Kazama F. 2007 – Assessment of surface water quality using multivariate statistical techniques: A case study of the Fuji river basin, Japan – Environ. Modell. Softw. 22: 464-475.

Simeonov V., Stratis J.A., Samara C., Zachariadis G., Voutsa D., Anthemidis A., Sofoniou M., Kouimtzis T. 2003 – Assessment of the surface water quality in Northern Greece – Water Res. 37: 4119-4124.

Simeonov V., Simeonova P., Tsitouridou R. 2004 – Chemometric quality assessment of surface waters: two case studies – Chem. Eng. Ecol. 11: 449-469.

Singh K.P., Malik A., Mohan D., Sinha S. 2004 – Multivariate statistical techniques for the evaluation of spatial and temporal variations in water quality of Gomti River (India): a case study – Water Res. 38: 3980-3992.

Singh K.P., Malik A., Sinha S. 2005 – Water quality assessment and apportionment of pollution sources of Gomti river (India) using multivariate statistical techniques: a case study – Anal. Chim. Acta 538: 355-374.

van den Heuvel M.R., Landman M.J., Finley M.A., West D.W. 2008 – Altered physiology of rainbow trout in response to modified energy intake combined with pulp and paper effluent exposure – Ecotox. Environ. Safe. 69: 187-198.

Wade J.W., Omoregie E., Ezenwaka I. 2002 – Toxicity of Cassava (*Manihot esculeta* Crantz) effluent on the Nile Tilapia, *Oreochromis niloticus* (L.) under laboratory condition – J. Aquat. Sci. 17: 89-94.

Wahbi O.M., Shalaby S.M., El-Dakar A.Y. 2004 – Effects of pulp and paper industrial effluent on some blood parameters, gonads and flesh proteins in experimentally exposed striped seabream *Lithognathus mormyrus* – Egypt. J. Aquat. Res. 30: 25-42.

Wilson J.Y., Kruzynski G.M., Addison R.F. 2001 – Experimental exposure of juvenile Chinook (*Oncorhynchus tshawytscha*) to bleached kraft mill effluent: hepatic CYP1A induction is correlated with DNA adducts but not with organochlorine residues – Aquat. Toxicol. 53: 49-63.

Wunderlin D.A., Diaz M.P., Ame M.V., Pesce S.F., Hued A.C., Bistoni M.A. 2001 – Pattern recognition techniques for the evaluation of spatial and temporal variations in water quality. A case study: Suquia river basin (Cordoba, Argentina) – Water Res. 35: 2881-2894.