Residual pollutants in treated pulp paper mill wastewater and their phytotoxicity and cytotoxicity in *Allium cepa*

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Abstract

Discharged pulp and paper mill wastewater (PPMW) were collected near M/s K. R. pulp and papers Limited, Shahjahanpur, India. Chemical analysis of the wastewater showed high BOD (3653-4180 mg L^{-1}) and COD (17890-19100 mg L^{-1}) values from two different sampling sites. The levels of total phenol were in the range of 389-432 mg L^{-1}, nitrogen (125-234 mg L^{-1}), sulfate (1926-2098 mg L^{-1}), chloride (3.12-5.43 mg L^{-1}) and lignin (38950-39000 mg L^{-1}) along with various heavy metals (Fe, 87-79; Zn, 34-22; Cu, 3.28-2.57; Cd, 1.90- 0.36; Ni, 6-5, and Pb, 41.23-36.54 mg L^{-1}); these were above the permissible limit as recommended by the CPCB and the USEPA. The BOD/COD ratio was <0.2 which indicated very low biodegradability of the organic matters present in the effluent. The organometallic complex generated from the pulp and paper industry persists in the environment and might toxic to the aquatic organism. The organic polymers, lignin, metals and ions present in the PPMW were characterized using SEM, EDAX,

FTIR and UV-Vis spectroscopy. The major pollutants detected included: nonacosane, heptacosane, octadecanoic acid, hexadecane, and 6-benzamide- 3- [2- [1-(phenylmethyl)-4-piperidinyl] ethyl] -1, 2-benzisoxazole; as well as a group of plant fatty acids classified as EDCs, and mutagenic pollutants. The cytotoxic and androgenic properties of these complex organics were examined using the seed germination test with *Phaseolus mungo* and cytotoxicity test with *Allium cepa*. Results showed that at >20% concentration of PPMW, α-amylase production was inhibited and chromosomal segregation at metaphase and anaphase during cell division was disturbed which resulted in c-mitosis, sticky chromosomes, and laggard chromosomes. In addition, SEM of the root of *A. cepa* showed fissures and fractured tissues of the root cap, probably due to the inhibition of auxins that were responsible for root cap formation. The findings indicated *A. cepa* as a good test model for examining the DNA damage and cytotoxicity by PPMW; and the discharged effluent should be treated at the tertiary stage for environmental protection.

Keywords: Antioxidants; Chlorolignin; Chromosomal aberration; Heavy metals; Phytotoxicity

1. Introduction

Pulp and paper mill wastewater (PPMW) is a major source of environmental pollution as they contain high levels of chlorinated compounds, chlorolignin, chlorinated hydrocarbons along with resin acid, tannin acid, phenolics, lignosulphonic acids, various surfactants, plasticizers, biocides, waxes, fatty acids, other complex organic compounds, heavy metals and inorganic compounds (Pokhrel et al. 2004; Chandra et al. 2012). In addition, several potentially toxic compounds such as dibenzo-p-dioxins and dibenzofurans are unintentionally generated during paper production and processing and discharged into the effluents. Several researchers have identified more than 200 organic and 700 inorganic compounds in pulp paper effluents (Suntio et al. 1988; Lacorte et al. 2003). These compounds increase the toxicity of the effluent as well as the chemical oxygen demand (COD), biological oxygen demand (BOD), and total dissolved solids (TDS) of the receiving aquatic resources that could imbalance aquatic life. Globally, fish toxicity has been observed in different aquatic resources that received PPMW (Singh and Chandra, 2019). These findings showed delayed sexual maturation, cellular damage and adverse effects on biochemical parameters in fish due to oxygen depletion and anoxia (Maria et al. 2002). The masculinization and negative impact observed in the reproductive system of western mosquito fish (*Gambusiaaffinis*) were due to the presence of androstenedione in the PPMW. The study detected detrimental influences on health, development and reproduction in both male and female *G. affinis* (Hou et al. 2018). In addition to the toxic effect on fish, other adverse effects were also reported in microorganisms, microplankton and benthic organisms that could reduce the self-purification capacities of rivers and other aquatic resources (McMartin et al. 2002; Karrasch et al. 2006). The toxic effects of PPMW were also noted in the terrestrial ecosystem (Iqbal et al. 2013). The effect

of pulp and paper mill effluent disposal on soil showed a high levels of accumulation of different metals in cellulose fibers that adversely affected the soil characteristics (Kumar et al. 2015). Haung et al. (2015) observed acute toxicity in *Photobacterium phosphoreum* induced by pulp and paper mill wastewater, where there was a direct correlation between COD and the soluble chemicals. A complex mixture of organic and inorganic compounds as residual recalcitrant pollutants present in PPMW was detected even after secondary treatment (Yadav and Chandra, 2018; Chandra et al. 2018). These compounds contribute to the toxicity and increase COD, some are carcinogenic and mutagenic, and others exhibit endocrine-disrupting properties. Along with their metabolic constituents, they could disturb the food chain and adversely affected human health (Ali et al. 2001; Savant et al. 2006; USEPA, 2012; Gustavo et al. 2015; Yadav and Chandra et al., 2018). Chemical analysis of the discharged pollutants indicated that the operating treatment processes in the industry were not able to degrade these residual pollutants. Hence, it is essential to fully identify the pollutants present in the treated effluent prior to its final discharged into a watercourse, and understand their toxic behavior for environmental safety. In India, there are more than 800 pulp and paper industries; out of these, 45 are large industries that manufacture writing papers and use the pulping and bleaching process described by the central pulp paper research institute (CPPRI, 2016). In general, 100-190 m^3 of wastewater is discharged per ton of paper production, reflecting the magnitude of the environmental problem caused by the pulp paper industry in India (Lindholm-Lehto et al. 2015). Further, due to the differences in the processing methods and raw materials for pulping, the discharged pollutants varied from industry to industry. The complete chemical characteristic of residual pollutants present in discharged pulp paper mill effluent was lacking. Therefore, this study focused on the novel solvent-based methods to extract and identify unknown compounds in the pulp and paper mill effluent and to evaluate the toxicity of these compounds

using *Phaseolus mungo* and *Allium cepa* as test models to understand the impact of PPMW on the ecosystem.

2. Materials and Methods

2.1. Site selection and sample collection

Sample collection was carried out in two selected points from the discharged site: upstream (wastewater-1) and downstream (wastewater-2) of M/s K. R. Pulp Papers Limited, located in Shahjahanpur, India (27°50'31.8"N 79°51'15.7"E) (Fig.1). The industry manufactures white paper, paperboard, through the Kraft process involving continued multi-stage of chlorinated pulping and bleaching process using eucalyptus, bagasse, and residue of sugar cane (100-200 tons per day). The collected samples were transported to the laboratory keeping at 4°C to maintain the original properties of pollutants present in the effluent. The analysis of all physico-chemical parameters and the extraction process of all organic compounds were carried out within 24 h. Three samples were collected from each site and on two separate seasons in May and November 2018.

2.2. Physico-chemical analysis of PPMW

The Physico-chemical parameters were analyzed as per standard methods (APHA, 2012). The pH, TSS, TDS, BOD, COD, electric conductivity (EC), levels of chloride, sodium, and potassium of the PPMW were measured using a selective ion electrode (Thermo Orion, USA, Model 960). The lignin and chlorophenols contents were estimated according to the method described by (Chandra et al. 2009). For heavy metal analysis, the samples were digested in 2% HNO_3 (APHA 2012) and analyzed by inductively coupled plasma (ICP-MS) spectrophotometry (Thermo Electron; Model IRIS Intrepid II XDL, USA) (Chandra et al. 2018). Soluble pollutants present in PPMW was detected by scanning the absorption spectrum in the range of 200-700nm

using a UV-Visible spectrophotometer (Thermo Fisher, Evolution 201, USA) (Yadav and Chandra, 2018).

Fig.1: Location map of M/s K. R. Pulp Papers Limited, located at Shahjahanpur, India (a). Sample is collected from two different sites i.e. wastewater-1 (upstream-near the industry) and wastewater-2 (downstream-away from 1.5 km)

2.3. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX)

For scanning electron microscopy, 5 mg air-dried PPMW was spotted on high purity aluminum stubs and analyzed as described by (Yadav and Chandra, 2018). The samples were placed on the aluminum stub and coated with platinum by means of a sputter coater (SC 7620 Mini Sputter Coater, Quorum Technology Ltd., UK). For elemental analysis of the PPMW, a specific point was chosen and elements examined through a high-resolution SEM equipped with an EDAX system (JEOL, JSM 6490LV, Japan).

2.4. FTIR analysis of PPMW

The FTIR analysis of the organic pollutants present in the effluent was carried out based on the method described by (Ladwani et al. 2016) using an FTIR Spectrophotometer-400 (Perkin Elmer, UK).

2.5. Extraction of residual organic pollutants

Different organic solvents i.e. ethyl acetate, isopropyl alcohol, n-hexane, and methanol were tested to compare the optimum extractability of the remaining residual organic pollutants in the PMWW and ethyl acetate showed the 70% extraction yield for the pollutants. Residual organic pollutants present in the PPMW collected from both sites were extracted and examined by the method described by (Chandra et al. 2009). The detected residual organic pollutants were identified by comparing their mass spectra (m/z) with the compounds provided in the NIST library, which was supplied with the instrument.

2.6. Phytotoxicity and genotoxicity evaluation of PPMW on P. mungo and A. cepa

Phytotoxicity evaluation of PPMW was carried out based on the protocols of the seed germination in *P. mungo* L. and *A. cepa* and root length bioassay recommended by the Organization for Economic Co-operation and Development (OECD, 2003). The genotoxicity assessment in *A. cepa* such as chromosomal break, abnormal cell cycle, and chromosomal fragments was performed as described by (Yadav and Chandra, 2018). For toxicity evaluation, different concentrations of the PPMW i.e. 20, 40, 60, 80 and 100% were used along with tap water as the control.

2.7. Effect of PPMW on α-amylase and molecular weight determination of enzyme

The toxicity was also expressed in terms of % inhibition on *α-amylase* activity of *P. mungo* treated with different concentrations i.e. 20, 40, 60, 80, and 100% (v/v) of PPMW as described by (Bharagava and Chandra, 2010). For enzyme precipitation, PPMW and tap water treated seeds were crushed with 60% wt/v; the pattern of amylase bands was determined using denaturing SDS-PAGE performed on a 10% polyacrylamide gel. The bovine albumin (67 kDa) was purchased from Sigma-Aldrich, USA, which was used as standards. The protein bands were stained with Coomassie Brilliant Blue R-250 and bands were observed in a gel documentation scheme (UViTECGeNei, USA) after destaining.

2.8. Scanning electron microscopy (SEM) of the treated root of A. cepa

Apical parts of the roots grown in the absence and presence of PPMW (100%) were cut and fixed with intermittent stirring in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4°C for overnight (12hrs) (Ahmed et al., 2015). The samples were dehydrated with acetone and CO_2 before visualization under JSM 6490LV SEM (JEOL, Japan) at an accelerating voltage of 10 kV.

2.9. Root cell viability test of A. cepa *by 2, 3, 5-triphenylte tetrazolium chloride (TTC) Assay*

Metabolically active and inactive root cells under the exposure of 20, 60, 80, and 100% of PPMW were examined using the method of (Shaymurat et al. 2012). The red-colored root tips were deemed viable, while others were either non-viable or dead.

2.10. Histological observation of A. cepa

Root segments (~2.0 mm) of *A. cepa* were cut and quickly immersed in H_2S saturated water as pretreatment for 30 min at room temperature (Khan et al. 1984). The section was prepared

using an ultra-microtome (CryoLeice EM UC7, Leica Microsystem, and India) and examined using a TEM (FEI Tecnai™ G2 Spirit Twin, Hillsboro, USA) at an accelerating voltage of 80KV.

2.11. Statistical analysis for data variability

All the experiments were carried out in triplicates and the data were reported as the standard deviation in physico-chemical analysis parameters. All the data were subjected to Tukey's analysis (Ott, 1984) using the Graph Pad software (Graph Pad Software, San Diego, CA.). One-way analysis of variance (ANOVA) was calculated for chromosomal aberration assay in *A. cepa* root cell exposed to PPMW at 50 and 100% concentration after 24hr exposure using SPSS software (20.0, 2011)

3. Results and Discussion

3.1. Physico-chemical properties and UV-Vis scanning spectra

The physicochemical analysis detected very high concentration of pollutants that were above the permissible limit even after the secondary treatment (Table1) (USEPA, 2002). PPMW collected from upstream (site-1) of the discharged drain was alkaline and had high BOD (3653-4180 mg L^{-1}) and COD (17890-19100 mg L^{-1}) values. The alkalinity of the samples might be due to the presence of residual content of sodium hydroxide and sodium sulfide utilized in the pulping process of the industry (Yadav and Chandra, 2018). The high-value COD and organic content were due to the release of various wood extracts along with chemicals used in the pulping process that resulted in the formation of complex compounds in the effluent (Huang et al. 2015; Muller et al. 2019). The five days BOD/COD ratio of the upstream and downstream PPMW was 0.21 and 0.20, respectively. This revealed the low biodegradability of organic pollutants present in the discharged wastewater. In addition, the high value of phenols, lignin, chlorophenols, chloride, and other salt

was noted in the discharged wastewater (Table 1). This confirmed that the secondary treatment process in the industry was not adequate to degrade all complex pollutants present in the effluent. Similar observations regarding the discharge of recalcitrant pollutants of the biologically treated effluent were reported previously (Chandra and Abhishek, 2011; Chandra et al. 2018; Yadav and Chandra, 2018). The discharged wastewater also showed the presence of various heavy metals beyond their prescribed environmental safe limits as reported by (Kumar et al., 2015). A significant amount of Fe (87-79 mg L^{-1}), Zn (34-22 mg L^{-1}), Cu (3.28-2.57 mg L^{-1}), Cd (1.90-0.36 mg L^{-1}), Mn (16-15 mg L^{-1}) and Ni (6-5 mg L^{-1}) were detected in the PPMW which are hazardous to the environment. This observation corroborated with previous findings (Chandra et al. 2011; Madan et al. 2018). The heavy metal in PPMW might be a result of metal leaching of the iron pipes when the corrosive alkaline black liquor (generated during wood digestion) passes through them. Zinc and Cu are known to be vital in the aquatic organisms due to their role in several biochemical mechanisms, but they become detrimental when present in high concentrations along with residual organic compounds (Chandra and Abhishek, 2011; Chandra et al. 2017; Yadav and Chandra, 2018). The discharged effluent can also become a source of groundwater contamination in the vicinity (Nurmesniemi et al. 2005). The inclusion of heavy metals into food chains could result in their accumulation in aquatic organisms and influence their biological and physiological mechanisms. As metals could be carcinogenic, they may also pose a serious threat to human health (Chowdhary et al. 2000; Smith et al. 2000; Ahmad et al. 2006; Farag et al. 2006; Singh and Chandra, 2019). Moreover, hazardous metals lead to the formation of reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals (Verma et al. 2008). Most of the heavy metals persist in water and sediments cause oxidative stress in fishes (Afshan et al., 2014). In addition, metals can disrupt the enzyme function due to their

might affinity toward sulfur, the carboxylic acid (-COOH) and amino ($-NH_2$) groups of protein that disturb transport processes (Slavin et al. 2017).

Table 1: Physico-chemical characteristics of discharged waste from pulp paper industry and their heavy metals content collected from M/s K. R. Pulp Paper Ltd. *Shahjahanpur,* Uttar Pradesh, India.

SN	Parameters	Wastewater site-1	Wastewater site-2	Permissible limit (EPA 2002)
1.	pH	8.4±0.42	7.9 ± 0.47^{ns}	5-9
2.	Colour	2345±143	2100 ± 105^{ns}	Dark Brown
3.	Total solid (TS)	1968±162	1611 ± 102^{ns}	-
4.	Total dissolved solid (TDS)	1789±42.56	1560 ± 31.25^{ns}	-
5.	Total suspended solid (TSS)	73±2.30	51 ± 3.21^{ns}	35
6.	Chemical oxygen demand (COD)	19100±754	17890 ± 821^{b}	120
7.	Biological oxygen demand (BOD)	4180±209	3653 ± 285^{b}	40
8.	Electrical conductivity (EC)	1762 ±86	1400 ± 84^{c}	1000
9.	Total Phenols	432±43.90	389 ± 22.23^{ns}	0.50
10.	Total nitrogen	234±5.76	125 ± 4.10^{ns}	143
11.	Sulfate	2098±89	1926 ± 97^{b}	250
12.	Phosphorus	173±5.98	150 ± 5.84^{ns}	200
13.	Cl^-	5.43±0.40	3.12 ± 0.20^{ns}	1500
14.	Na^+	331±15.87	294 ± 14.20^{ns}	200
15.	K^+	19.05±0.70	17.8 ± 0.80^{ns}	-
16.	Lignin	39000±1110	38950 ± 1124^{ns}	-
17.	Chlorophenol	431±12.76	311 ± 10.23^{a}	3.0
	Heavy metals			
18.	Iron (Fe)	87 ±1.89	79 ± 1.48^{b}	2.00
19.	Zinc (Zn)	34±1.35	22 ± 1.40^{a}	2.00
20.	Copper (Cu)	3.28±0.17	2.57 ± 0.19^{c}	0.50
21.	Cadmium (Cd)	1.90±0.09	0.36 ± 0.02^{a}	0.01
22.	Manganese (Mn)	16±0.77	15 ± 0.57^{ns}	0.20
23.	Nickel (Ni)	6±0.38	5 ± 0.34^{a}	0.10
24.	Chromium (Cr)	4±0.09	3 ± 0.08^{a}	-
25.	Lead (Pb)	41.23±2.15	36.54 ± 1.54^{c}	-

All the values are means of triplicate (n=3) ±SD. Unit of all parameters are in mg L^{-1} except pH, colour (Co-Pt Unit) and EC ($\mu mhoscm^{-1}$). ^{a}= highly significant at $p<0.001$, ^{b} =significant at $p<0.01$, ^{c}=less significant at $p<0.05$ and ^{ns}= non-significant at $p>0.05$, between samples collected from site-1 and site-2.

The physico-chemical analysis of downstream also revealed the high concentration of Na^+ (331-294 mg L^{-1}) and K^+ (19.5- 17.8 mg L^{-1}) that are beyond the permissible limit as shown in Table 1. The high concentrations of Na^+ and K^+ influenced the salinity of the effluent and this could cause aquatic toxicity and soil pollution. The higher electrical conductivity values might be due to the high salt and ions content of PPMW (Deepali et al. 2009). Chloride present in PPMW is found to be more toxic than sulfates to flora and fauna including the microbial community. Therefore, the pulp paper mill effluent was unsuitable for irrigation (Reddy and Rao, 2001). The effluent discharge from the pulp paper industry is also toxic to phytoplankton and macro-invertebrate, thus can affect the food chain of the aquatic ecosystem (Ojunga et al. 2010; Gauthier et al. 2001).

The presence of various soluble pollutants and biologically impervious organic compounds in the effluent has contributed to the PPMW toxicity. The high TDS may be due to the presence of dissolved lignocellulosic particles along with fine fibers and pith particles. The lignin contents were noted to be very high (39000-38950 mg L^{-1}) in the effluent, this might be the source of the dark color (2100-2345 co.pt) of the PPMW. The dark color due to lignin with high TDS of PPMW adversely affected aquatic flora and fauna along with microorganisms by increasing the pollution parameters (BOD, COD, organic pollutants). Besides, the combination of heavy metals with organic compounds aggravates the toxicity and complexity of pollutants (Yadav and Chandra, 2018). The detection of the complex pollutants in the discharged effluent suggested these compounds are recalcitrant and cannot be degraded easily by the microbial community in the treatment plants (Haq et al. 2017; Chandra et al. 2018). The growth of *Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp. and *Citrobacter* spp. directly correlate with the ligninolytic pollutants

present in pulp paper mill wastewater (Afroz and Singh, 2014; Gauthier and Archibald, 2001). *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp. were also noted in river water receiving the discharged pulp paper mill effluent in India (Chandra et al., 2006). PPWW collected from downstream (site-2) of the discharged drain showed a slight decrease in all pollution parameters. This reduction might be due to a number of factors: firstly, some pollutants may settle during their runoff through the discharge drain; secondly, biodegradation or biotransformation may occur as a result of the joint action of natural plants and microbes growing along with the discharge drain. The in-situ bioremediation of pollutants during the run-off had been reported in other types of effluents (Yadav and Chandra, 2018) and PPMW from other sites (Chandra et al., 2017; Chandra et al., 2018).

3.2. EDAX analysis and Surface view of pollutants present in PPMW

The EDAX analysis was performed to determine the elemental constituent in PPMW (Fig. $2a_1$ and b_1). Fig. $2a_1$ and b_1 indicated that the iron (12.10%, 11.43%), oxygen (70.44%, 64.32%), and silica (16.30%, 12.85%) content was in considerable amount followed by manganese and potassium. The high content of aluminum might be due to the aluminum stub used for the gold coating of the sample. The images of SEM analysis of PPMW upstream and downstream samples are presented in Fig. $2a_2$ and b_2.The SEM images indicated that the PPMW had irregular constituents that provided a large surface area for the adsorption of various pollutants along with heavy metals and lignin compounds. The un-derivatized lignin showed a granulated structure with grains or oval particles of lightweight in different sizes. The shapes crystal showed the presence of different heavy metals, various particles and lignin (Liu et al. 2013; Batista et al. 2018). Similar to the physico-chemicals results, the UV scanning spectra of upstream PPMW and downstream

PPMW showed the presence of organics and nitrates in the wastewater as indicated by the presence of absorption maxima between 270 to 310nm and > 250nm, respectively (Fig. 2c and d). The upstream PPMW displayed different stable peaks ranging from 270 to 450 nm, while downstream PPMW has different stable peaks ranging from 220 to 350 nm, respectively. The formation of various peaks in the UV and visible region indicated the presence of a complex mixture of organometallic compounds in upstream and downstream PPMW (Yadav and Chandra, 2018). Some peaks were also observed after 380 nm in both samples, which showed the turbidity of the effluent. This study confirmed that PPMW is not safe for irrigation, human, and animals.

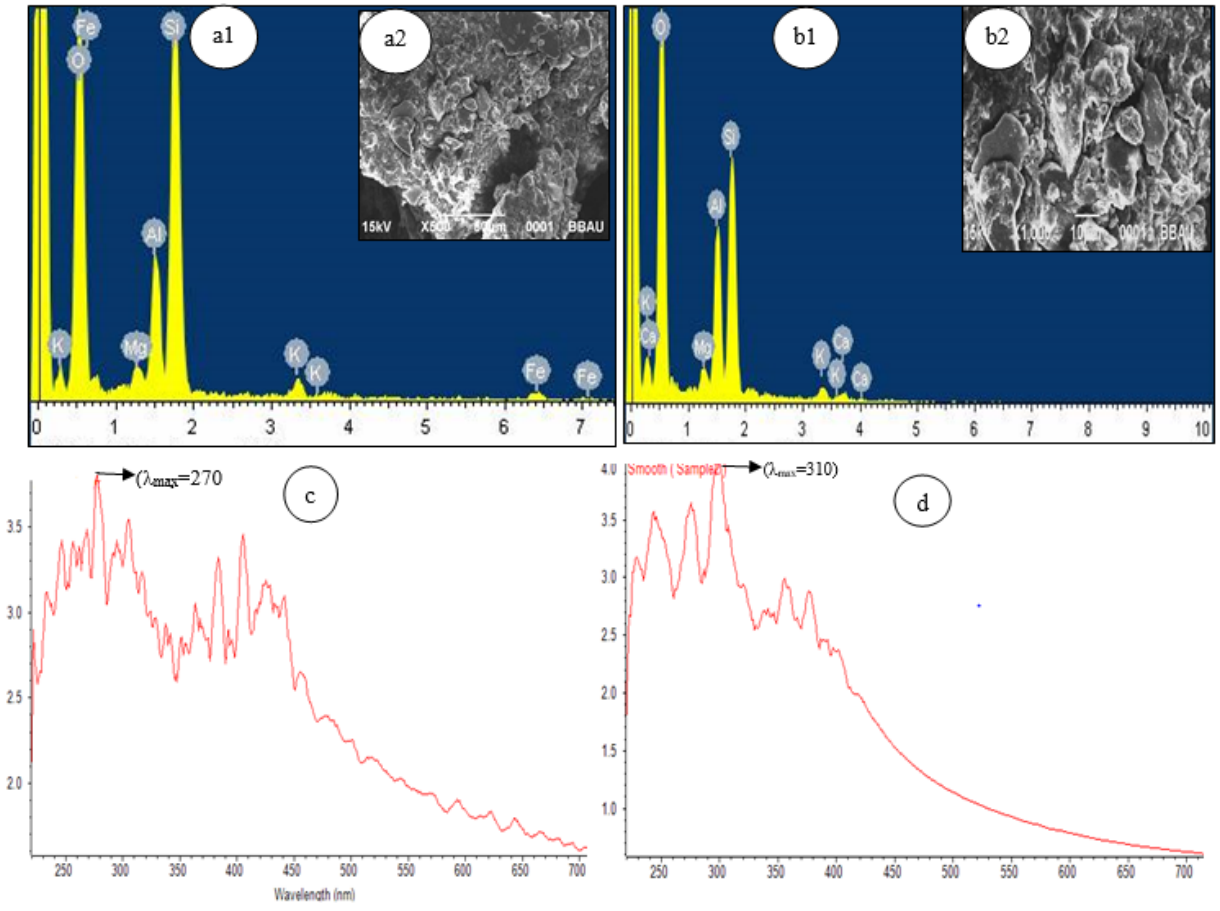


Fig.2: EDX, scanning electron microscopy of PPMW collected from site 1 (upstream; a_1, a_2) and site 2 (downstream; b_1, b_2). UV-Vis spectra of PPMW, upstream (c) and downstream (d)

3.3. FTIR analysis

FTIR spectral analysis covered spectra cover a wide range of functional groups with strong and weak bonds of organic compounds and polymers from upstream and downstream PPMW. The average spectra in the 4000-500 cm^{-1} wave number region showed strong intensity at 3408.9, 2923.0, 1644.8 and 1430 cm^{-1} in upstream collected samples as shown in (Fig. 2e), while downstream PPMW showed high intensity at 3436.4, 2923.8, 1631.9, 1428.1 cm^{-1} (Fig 2f). The peaks at 3408.9, 2923.0, 1644.8, 1430 cm^{-1} in upstream were related to the functional group of O-H as alcohols and organic acids (3408.9); asymmetric aliphatic C-H stretch variation methylene (CH_2)/Alkene C-H stretching (2923); aromatics (1644.8) and Aromatic C=C stretching variation (1430), respectively. Absorption 3436.4, 2923.8, 1631.9 and 1425.8 cm^{-1} in downstream PPMW showed O-H as alcohol and phenol bonds; alkanes C-H, alkyls C=C and alkene; amide, ketone, and quinone, C=O stretching vibration in conjugated carbonyl of lignin and a carboxylic acid, respectively. The presence of these strong bonds might be due to the vibration of various groups of organic acids, fatty acids, alkane, aromatic organic compound, polycyclic aromatic hydrocarbon, proteins, lipids, carbohydrates and aromatic heterocyclic, showed the presence of complex organic pollutants in PPMW. Fatty acid in PPMW is a by-product of the pulping process during papermaking. Ether deformation is the grafting of epichlorohydrin onto the lignin from the pulp paper industry. The comparative analysis of both samples showed similar types of broadband i.e. 1025.9, 784.9, 681.6, 525.4, and 465. They showed the presence of similar types of the bond of amines; meta-disubstituted aromatics; alcohols and phenols; halogen compounds (chlorine compounds C-Cl) and cycloalkane that are non-degradable and persistent in nature even after traveling of PPMW. This finding also supported the outcome of the GC-MS analysis of PPMW

(Fig. 3). Moreover, FTIR analysis also indicated that some pollutants might be transformed as they were transported along with persistent pollutants.

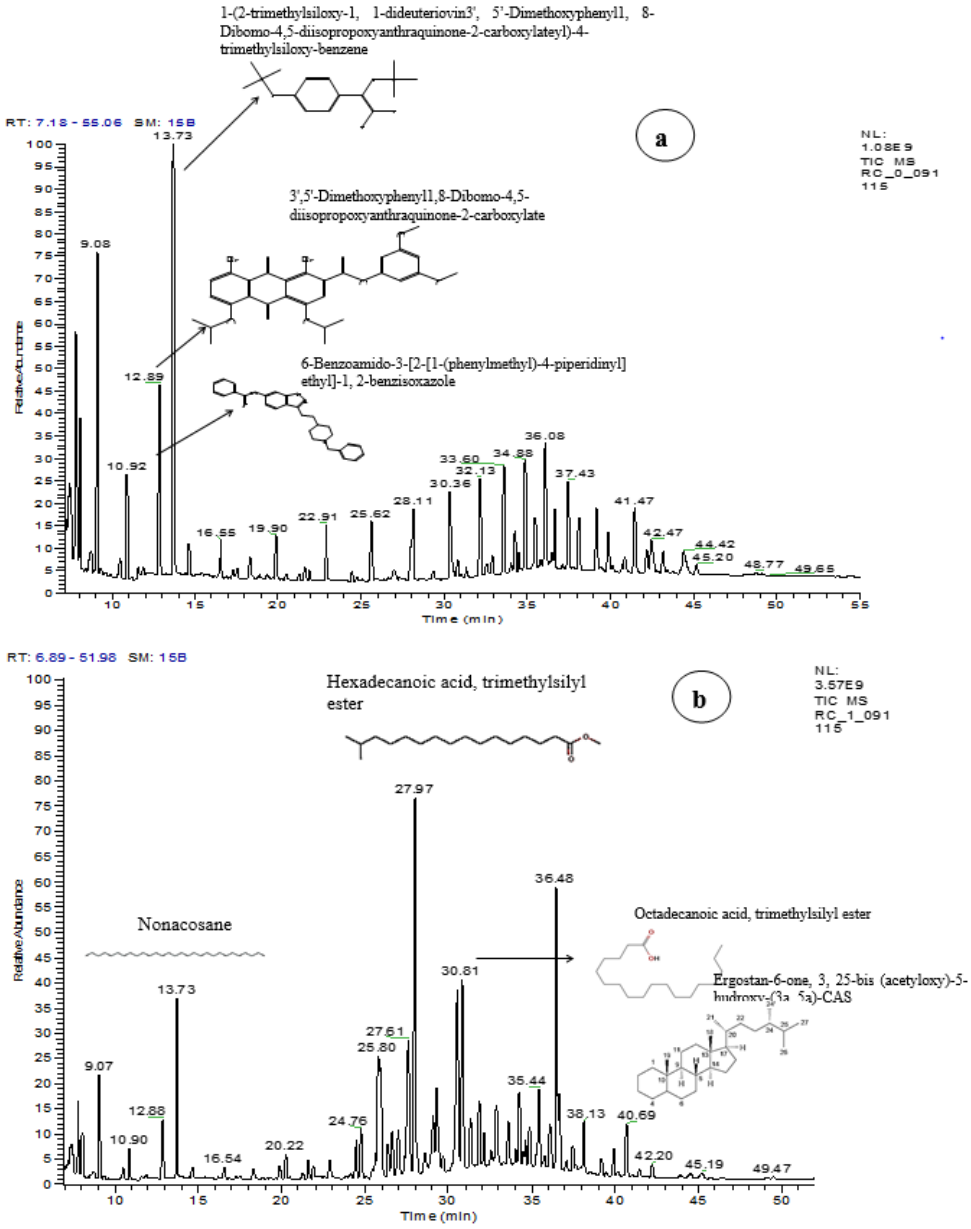


Fig.3: Total Ion Chromatogram (TIC) of TMS derivative detected residual Organic Pollutants from ethyl acetate extract of PPMW collected from site 1 (upstream): (a) and site 2 (downstream): (b)

3.4. Characterization of metabolites

The GC-MS chromatograms and the identification of the compounds were shown in Fig. 3a-b and Table 2, respectively. The GC-MS chromatogram of ethyl acetate extracted sample collected from upstream showed three dominant peaks at RT-9.08, 12.89, 13.73 as shown in Fig. 3a. These compounds showed >90% similarities with the compound reported in the NIST library available with instruments. The compound identified at RT-9.08 as heptacosane with similarity 91.10 and molecular weight 380.745 g/mol^{-1}. While IInd at RT-12.09 was identified as 4, 5 di-isopropoxy anthraquinone-2-carboxylate showed similarity 92.50 with the compound available in the NIST library with molecular weight 252.22 g/mol^{-1} and is a polycyclic aromatic hydrocarbon compound. This compound induced apoptosis in rat hepatocytes based on their chemical composition (Chandra and Kumar, 2017; Kagedal et al. 1999). The most predominant peak at RT-13.73 with a similarity index of compounds in the NIST library was identified as nonacosane with a molecular weight of 408.6 gmol^{-1}and a plant fatty acid. However, its metabolic product has been associated with the androgenic activity (Hankin and Kolattukudy, 1968). This compound has been detected previously in pulp paper mill effluent discharged after secondary treatment (Yadav and Chandra, 2018). The fatty acid compounds of plant origin were predominant in the discharged wastewater and the bacterial community in the effluent treatment plant could not degrade these pollutants. This might be due to either the bactericidal or bacteriostatic effect of the pollutant (Taylor et al. 1981). As the ability of the bacterial community to degrade these compounds was affected adversely, then on-degraded compounds were released into discharged effluent. Some minor peaks were detected at RT- 10.92, RT- 37.43 2,5-Bis(methylthio)-3-phenyl-7-(6-phenyl-1,3,5-hexatrienyl)pyrazole [1,5-a] pyrimidine, RT-2-morpholino ethane sulfonic acid with molecular weight 195.24 gmol^{-1}, and RT- 44.42 O-trimethyl silylated (d13)-phe-glu-a identified as 6-

benzamide-3- [2-[1- (phenylmethyl)-4-piperidinyl] ethyl] -1,2-benzisoxazole with similarity 43.30 51.10, 54.23 and 23.33 %, respectively. The lower similarity might be due to its complexity that is not listed in the NIST library. The identified compound reported here might provide new information as environmental pollutants that require further investigation for its ecological risk assessment. The peaks detected at RT-19.90, 28.11, 32.13 and 45.20 are identified as plant fatty acids conjugated with organic compounds as shown in Table. 2. These compounds might have been generated during the effluent treatment process and were subsequently transformed as new products via microbial activity (Yadav and Chandra, 2018). These compounds were found in benzene/ethanol extractive of eucalyptus (Peng and Wu, 2008). Hexadecanoic acid, (2-phenyl-1, 3-dioxolan-4-yl) methyl ester, cis, hexadecane, 2, 6, 10, 14-tetramethyl- (CAS), and Octadecane, 3-ethyl-5-(2-ethyl butyl)-(CAS) with molecular weight 256.43 $gmol^{-1}$, 226.41 $gmol^{-1}$and 254.5 g/mol^{-1}. The similarly with compounds listed in the NIST library was 95.20, 81.01 and 86.23%, respectively. Hexadecanoic acid (palmitic acid-C16) was the most abundant, thus it is in agreement with previous findings (Morrison et al., 2001). Their study showed that palmitic acid was the major fatty acid found in the extracts from fibers of several flux cultivars detected from downstream at RT-27.97 with 95% similarity. The synthesis of Hexadecanoic acid from the carboxylic group with C-14has been reported as a DNA fragmentation inducer in a human melanoma cell line and similar compounds were reported in previous studies (de Sousa Andrade et al. 2005; Gonzalez et al. 2000). Octadecanoic acid was detected in the PPMW samples; it has been reported as an antiquorum sensing molecule in bacterial metabolites and is responsible for aquatic toxicity (Singh et al. 2013; Chandra and Kumar, 2017). This finding also collaborated with previous data (Kaushik et al. 2010). However, in this study, the GC-MS spectra of downstream wastewater showed four major peaks at RT-13.73, 27.97, 30.81 and 36.48. Similar compounds were also found in the upstream

wastewater site-2 at RT-13.73 that showed 91.08% similarity with nonacosane. At RT 27.97 a compound is obtained which shows 95% similarity with Hexadecanoic acid, trimethylsilyl ester (molecular mass, 328.612 g/mol^{-1}). Tetradecanoic acid and Hexadecanoic acid are plant origin fatty acids that were detected in humic substances (Reveille et al. 2003). These compounds are classified as endocrine-disrupting chemicals by the (USEPA, 2012). The RT-30.81 showed a similarity of 94% with octadecanoic acid and trimethylsilyl ester. They caused apparent chromosomal aberrations in the treated biological samples including centromeric holes, chromatid breakage, attenuation, acentric fragments pycnosis, polyploidy, and chromosomal gaps (Firbas and Amon, 2014; Nefic et al. 2013). Phenolic compounds, acidic compounds (acetic acid, benzene acetic acid, hexadecanoic acid, octadecane, and octadecanoic acid) have been reported earlier during fungal peroxidase degradation of lignosulfonates and also from bacterial degradation of black liquor lignin (Chandra et al., 2012). Some minor peaks were obtained in samples collected from the PPMW (Fig. 3a-b). The GC-MS analysis of the samples collected from both sites showed the presence of N_2 containing aromatic heterocyclic organic compounds, alkanes, aromatic organics, polycyclic aromatic hydrocarbons, organic compounds, aromatic carboxylic acids, fatty acids, and phenolics, where most of the pollutants are toxic and mutagenic in nature. Stearic acid, 3-(octadecycloxy) propyl ester (CAS) at RT-34.88 with 78.20 % similarity with the compound listed in the NIST library, it is a saturated monobasic fatty acid with 18 carbon-chain lengths of 284.48 g/mol^{-1} molecular weight; this is also known as octadecanoic acid. Stearic acid can be readily chlorinated and converted into toxic compounds (Leach and Thakore, 1974) and the polymorphic forms of stearic acid are mutagenic compounds (USEPA, 2012). This finding corroborated with results reported previously by Yadav and Chandra (2018).The highly toxic organic pollutants can cause significant damage to body organs and systems, such as the nervous,

respiratory, circulatory, immune, reproductive, sensory and endocrine systems (Yadav and Chandra, 2018). In upstream PPMW, nonacosane (100%, RT= 13.73) was present in significant quantity followed by heptacosane (76%, RT=9.08), 6-(4-chlorophenyl)-2,5,5-triphenyl-5,8-6H-azeto[1,2a][1,3] thiazole [4,5-d] pyrimidine (58%, RT=7.23);3',5'-dimethoxyphenyl1,8-dibromo-4,5-di-isopropoxy anthraquinone-2-carboxylate (47%, RT=12.89); N-methylleuconolane (32%, RT=36.08);stearic acid, 3-(octadecyloxy) propyl ester (CAS) (38%, RT=34.88) etc. In the downstream PPMW, hexadecanoic acid, trimethylsilyl ester (RT=27.97) was present in 76% followed by 6-cyclopentyloxy-2, 3-bis (hydroxymethyl)-1-(2-chloro-4-pyridyl)-7methylnaphthalene (60%, RT=36.48), octadecanoic acid, trimethylsilyl ester (42%, RT=30.81), nonacosane (37%, RT=13.73), tetrabromo-2-(3-hydroxy-1,2-dihydro-quinol-2-ylidene)-2,3-dihydro-1H-benz[f] indene (67.10%, RT=10.90), ergostane-6-one, 3,25-bis (acetyloxy)-5-hydroxy-(3a,5a)-CAS (78%, RT=40.69); many of these chemicals are endocrine-disrupting chemicals (EDCs). EDCs are also known as exogenous agents that interfere with the synthesis, secretion, transport, action, and binding of natural hormones in the body that are responsible for the maintenance of development reproduction behavior and homeostasis of animals (USEPA, 2012). This study showed the detection of endocrine disruptors that share sufficient structural resemblance with endocrine hormones in PPMW, these compounds could interact with locations of animal endocrine receptors and cause adverse effects on reproductive success and long-term survival of delicate aquatic communities. Benzene acetic acid, 3, 4-tris [(trimethylsilyl) oxy]-, trimethylsilyl ester at RT-63.48 shared 89.10% similarity with the compound in the NIST library, was only detected from the downstream site. This compound was a residual complex compound reported in our previous studies (Chandra et al., 2018; Chandra and Singh, 2012). Ergostane-6-one, 3, 25-bis (acetyloxy)-5-hydroxy-(3a, 5a)-CAS at RT 40.67 has 68 % similarity with the

compound in the NIST library. It is a tetracyclic triterpene steroid due to the presence of two singlets and three doublets of the methyl groups, with molecular weight 386.7 g/mol^{-1}and has no known uses, but the most relevant of these are the strongly derivatized with anolides. The ester of benzoic acid (RT=16.55) was found in the PPMW of the upstream site suggests it might originate from the raw material used by the industry; benzoic acid naturally occurs in many plants and functions as an intermediate in the biosynthesis of several secondary metabolites. Benzoic acid can cause gastric pain, nausea, vomiting and possible allergic reactions (USEPA, 2012). A series of n-alkanes from C16 to C29 was also detected in the PPMW. Nonacosane (C29, RT=13.73) was the most abundant (37%) followed by Octadecane, 3-ethyl-5-(2-ethyl butyl)-(CAS) (RT=32.13, 25%) and hexadecane, 2, 6, 10, 14-tetramethyl-(CAS) (RT=22.91. Benzene acetic acid (RT=27.61) was also detected in the PPMW, it is a catabolite of phenylalanine as a precursor of lignin which remains in wood and discharge during the wood digestion and pulping processing. The phenolic compound such as 1-(5-ethyl-tetrahydrofuran-2-yl)-3, 3-dimethyl-butane-2-one was also found in upstream PPMW which has been reported as a highly toxic compound. This report indicated that major residual organic toxic compounds i.e. mutagens, carcinogens, and endocrine disrupters of the environment would have occurred in PPMW. The presence of several hydrocarbon pollutants in PPMW that is known to lead to several health and environmental impacts are of great concern for the environment and health.

Table 2a: Identified Residual Organic Pollutants by GC-MS in the TMS derivative ethyl acetate extracts of PPMW from site-1

RT	Identified compound (Site 1)	Compounds% similarity with NIST Library	Relative abundance	Nature of compounds	Toxicity
9.08	Heptacosane	91.10	76	Alkane	Aquatic and terrestrial toxicity
10.92	6-Benzoamido-3-[2-[1-(phenylmethyl)-4-piperidinyl]ethyl]-1,2-benzisoxazole	43.30	27	Aromatic organic compound	Data not available
12.89	3',5'-Dimethoxyphenyl1,8-Dibomo-4,5 diisopropoxyanthraquinone-2-carboxylate	92.50	47	Polycyclic aromatic hydrocarbon	Data not available
13.73	Nonacosane	90.08	100	Organic compounds	Oral, dermal and paraffin liver in cows
19.90	Hexadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis	95.20	13	Fatty acid	DNA damage, human fibroblasts
28.11	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	81.01	19	Alkane hydrocarbon	Irritation, CNS depression, and gastrointestinal tract irritation
30.36	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	74.22	22	Fatty acid	Gastrointestinal toxicity, eye and dermal irritation effects
32.13	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (CAS)	86.23	25	Alkane hydrocarbon	Respiratory tract irritation, skin & eye irritation.
34.88	Stearic acid, 3-(octadecyloxy) propyl ester (CAS)	78.20	28	Fatty acid	*Toxicity* in B and T cell lines, cancer, neurotoxicity, organ toxicity and irritation.
37.43	2,5-Bis(methylthio)-3-Phenyl-7-(6-phenyl-1,3,5-hexatrienyl)pyrazolo[1,5-a]pyrimidine	51.10	23	-	Fluorouracil toxicity
42.47	2-morpholino ethane sulfonic acid	54.23	08	-	Low acute toxicity
44.42	O-trimethysilylated (d13)-phe-glu-a	23.33	08	-	Low acute toxicity
45.20	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (CAS)	83.10	08	-	Data not available

Table 2a: Identified Residual Organic Pollutants by GC-MS in the TMS derivative ethyl acetate extracts of PPMW from site-2

RT	Identified compound (Site 2)	Compounds% similarity with NIST Library	Relative abundance	Nature of compounds	Toxicity
9.07	2,3-Dibromo-[1] benzopyrano [4,3-b]pyrrol-4(1H)-one	72.30	22	Aromatic heterocyclic	Carcinogenicity
10.90	Tetrabromo-2-(3-hydroxy-1,2-dihydro-quinol-2-ylidene)-2,3-dihydro-1H-benz[f]indene	67.10	07	-	EDCs, reproductive toxicity
12.88	3',5'-Dimethoxyphenyl1,8-Dibomo-4,5-diisopropoxyanthraquinone-2-carboxylate	53.30	13	-	Methemoglobinemia, acute, chronic inhalation, fatigue, weakness, dyspnea, headache
13.73	Nonacosane	85	37	Organic compounds	Oral, dermal and paraffin liver in cows
27.61	Benzene acetic acid, 3, 4-tris [(trimethylsilyl) oxy]-, trimethylsilyl ester	89.10	30	Organic compound	Chronic oral toxicity study of erythritol in dogs.
27.97	Hexadecanoic acid, trimethylsilyl ester	95	76	Saturated fatty acids	Sub chronic or chronic
30.81	Octadecanoic acid, trimethylsilyl ester	94	42	Saturated fatty acids	Acute toxic
36.48	6-Cyclopentyloxy-2, 3-bis (hydroxymethyl)-1-(2-chloro-4-pyridyl)-7-methoxynaphthalene	63	60	Organic compound	Harmful in contact with skin, acute toxicity, dermal
40.69	Ergostane-6-one, 3,25-bis(acetyloxy)-5-hydroxy-(3a,5a)-CAS	68	14	-	EDCs, genotoxicity and sub chronic toxicity EDCs, genotoxicity and sub chronic *toxicity*

3.5. Effect on seed germination

A seed germination test, the germination index (GI), that measures the effect on root elongation has been used as a rapid, reliable and reproducible technique to indicate the damaging effect of different industrial waste on plant growth (Yadav and Chandra, 2018). The responses of *A. cepa* and *P. mungo* to the toxicity of PPMW in terms of root length were different as shown in Fig. 4a. Root length of *A. cepa* (2.41 cm) and *P. mungo* (2.05 cm) treated with 20% PPMW was almost the same with their controls, measuring 2.50 and 2.22 cm, respectively. The lower concentration of PPMW may be growth-supporting due to the presence of one or more essential elements. At the concentration, above 20% PPMW, the root length of both crops showed a significant reduction (Fig. 4a). This might be due to a higher concentration of toxic organic pollutants that impair seed germination. High salt load and metal content can act as an inhibitor for plant hormones, i.e. amylases, auxins, gibberellins, and cytokinins that are essential for seed germination, seedling growth and plant development. This reduction was higher in *P. mungo* then *A. cepa*, indicating a higher sensitivity of *P. mungo* than *A. cepa.* The GI of *A. cepa* was 76.22, 43.15, 35.62, 16.73 and 4.74 for 20, 40, 60, 80 and 100% PPMW, respectively. The result showed a concentration-dependent GI reduction. Seed germination was reduced when exposed to a higher concentration of PPMW due to high osmotic pressure caused by high levels of salt in PPMW. (Ilic et al., 2015) also found the different concentrations of lead and the different degrees of permeability of seed coat led to a different degree of germination inhibition. Similarly, the seed germination index of *P. mungo* at different concentrations (20, 40, 60, 80 and 100%) of PPMW was 73.63, 36.24, 28.63, 13.84, and 2.85 respectively. A 9.06% decrease in GI was found at 20% PPMW, but a significant decrease ($p<0.01$) of 96.45% was noted at 100% of the PPMW

concentration in comparison to the control. These results clearly indicated that the increase in the toxicity of waste was directly related to the increase in the concentration of PPMW.

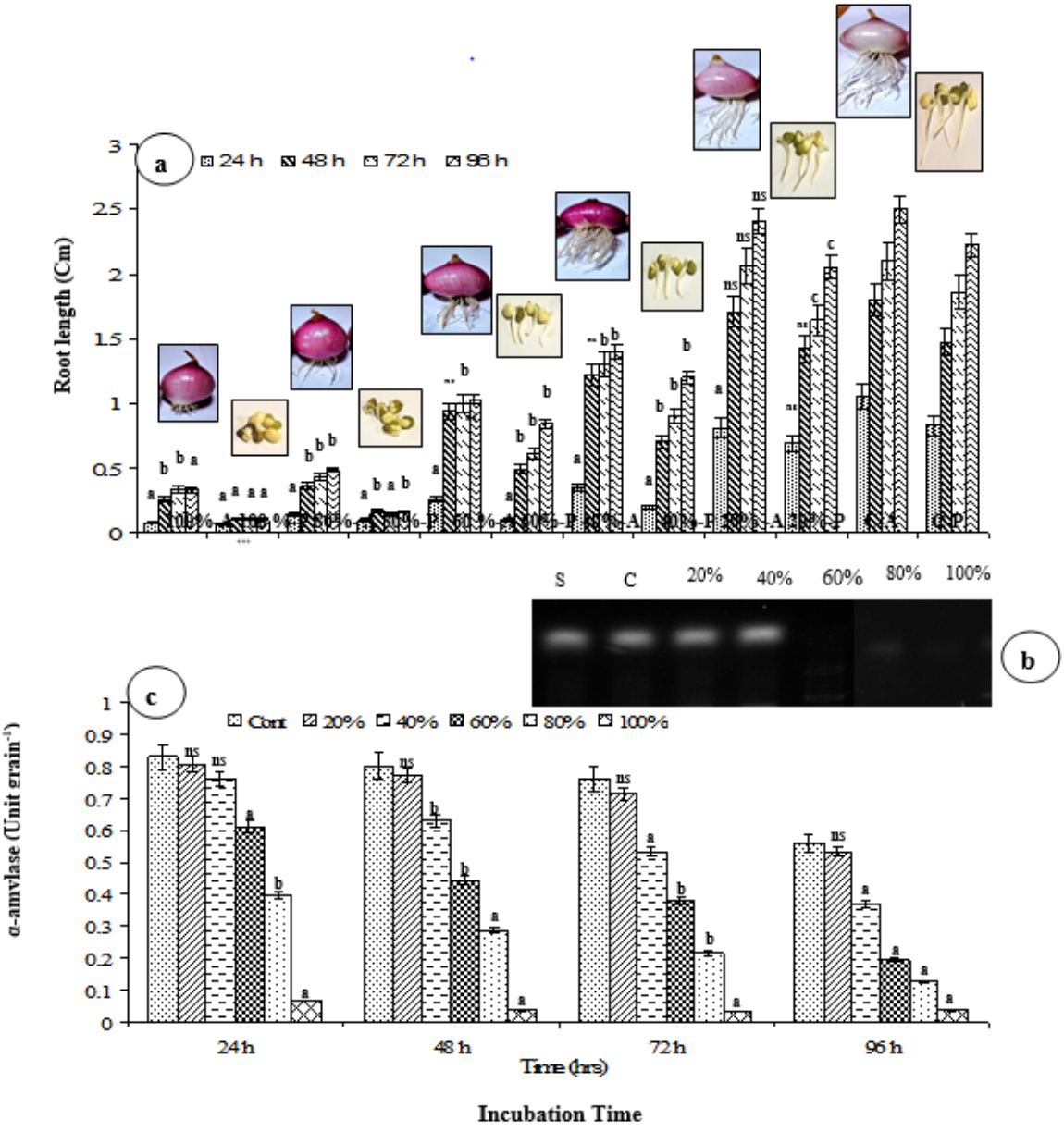


Fig.4: Effect of different concentrations of PPMW on root length (a) and α- Amylase activity in *P. mungo L* (b). Inserted figure (c) showed different band pattern of α-amylase on SDS PAGE of *P. mungo L*. treated with various concentration of PPMW. S: Standard amylase (bovine albumin; 67 kDa), C: control treated with tap water.

Statistical significant between the value of root length of each crops treated with different concentration of PPMW was evaluate with their respective control through ANOVA. Significant level a=P<0.001, b=P<0.01, c=P<0.05, ns=P>0.05.

3.6. Toxicity evaluation

The α-amylase activities were measured in the *P. mungo* treated with different concentrations of PPMW along with tap water as a control. The analysis showed the presence of a single α-amylase with an estimated molecular weight of 67 kDa (Fig. 4b). The intensity of the band decreased gradually as the concentration of PPMW increased as compared to the control. A very light band of α-amylase was observed in the seeds treated with 100% PPMW. Results showed that PPMW inhibited amylase activity and a continuous decline in α-amylase activity at a higher concentration of PPMW (Fig. 4b). In germinating seeds, starch degradation is initiated by α-amylase producing soluble oligosaccharides. These are then hydrolyzed by α-amylase into maltose and glucose providing energy to germinating seeds. The study showed 100% PPMW reduced the α-amylase activity to 0.037 unit g^{-1}, but the seeds treated with 20% PPMW showed similar α-amylase activity as the control (0.56 and 0.53 unit/g, respectively). It appeared to have a threshold level where the concentration of PPMW would affect the amylase activities; probably due to the presence of an optimum level of organic nutrients that are vital to sustaining plant growth. The decrease in amylase activity at higher PPMW concentration might be due to the high pollution content throughout seed germination that influenced multiple physiological and biochemical

processes. Toxic pollutants concomitantly affected amylase activity have also been documented in previous reports (Bharagava and Chandra, 2010; Fendri et al. 2013).

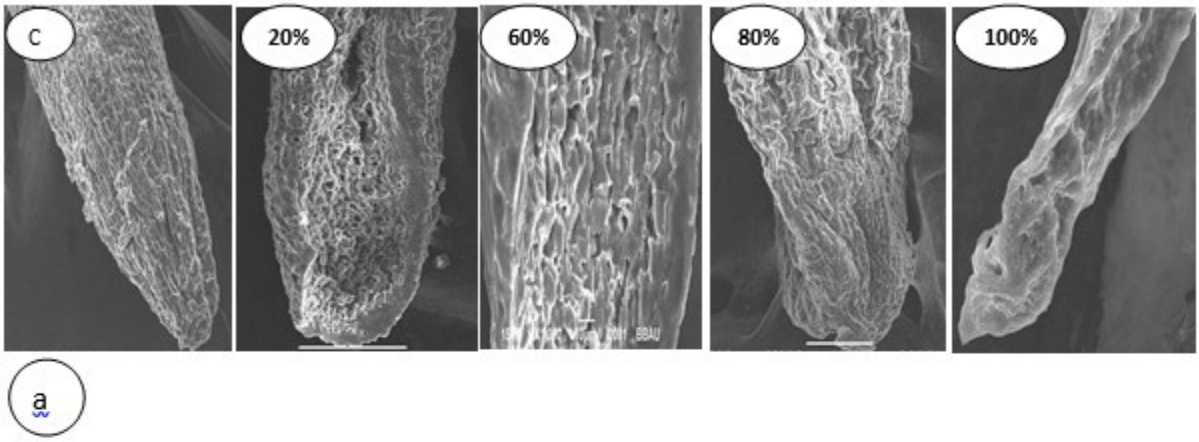
3.7. Cytotoxicity study by scanning electron microscopy

A. cepa showed noticeable morphological changes under SEM after being treated with 100% PPMW (Fig. 5a). The surface of the untreated root of the *A. Cepa* control had a clear, smooth, and intact root surface along with a normal root cap (Fig. 5a). Toxicity of heavy metal exposure on the root growth of *Zea Mays* L. related to their specificity and selectivity has been observed by (Ivanov et al., 2001). The inhibition of root elongation by different heavy metals in ryegrass (*Lolium perenne*) has been also reported previously (Wong and Bradshaw, 1982). Aberrations, fissures, and fractured tissues were noted on the surface of roots treated with different concentrations of PPMW (Fig. 5a). SEM observation also showed crumbled, shrink and ruptured root cells and damaged root cap after treatment with PPMW. The root of *A. cepa* showed similar abnormalities due to oxidative stress caused by ZnO nanoparticles (Ahmed et al. 2015). The observed abnormalities might be due to the presence of various types of carcinogenic, mutagenic, and androgenic compounds including heavy metals present in PPMW. All these observations suggested PPMW contained highly toxic and hormone-disrupting compounds.

3.8. TTC assay for root cell viability

Living tissues have the capacity to reduce the TTC (colourless) into Formazan (colored compound) by the enzyme dehydrogenases through H transfer during the respiration. Formazan is a non-diffusible stain so that living tissues have become red when incubated in the solution of this chemical. TTC tests showed a different pattern of cell viability after *A. cepa* was exposed to different concentrations of PPMW over a period of time (Fig. 5b). For the 12 h treatment, all root

tips were colored red including the control, indicating the cells were viable at initial stages of treatment. The viability of root reduced with time at all concentrations. All the roots were dead after 96 h exposure except for the control (Shaymurat et al. 2012) showed similar concentration-dependent phytotoxicity and genotoxicity of ZnO nanoparticles on *Allium sativum*. The non-viability of *A. cepa* tissues might be due to the pollutants present in PPMW that induced chromosomal aberrations and lead to mitotic arrest and cell death.



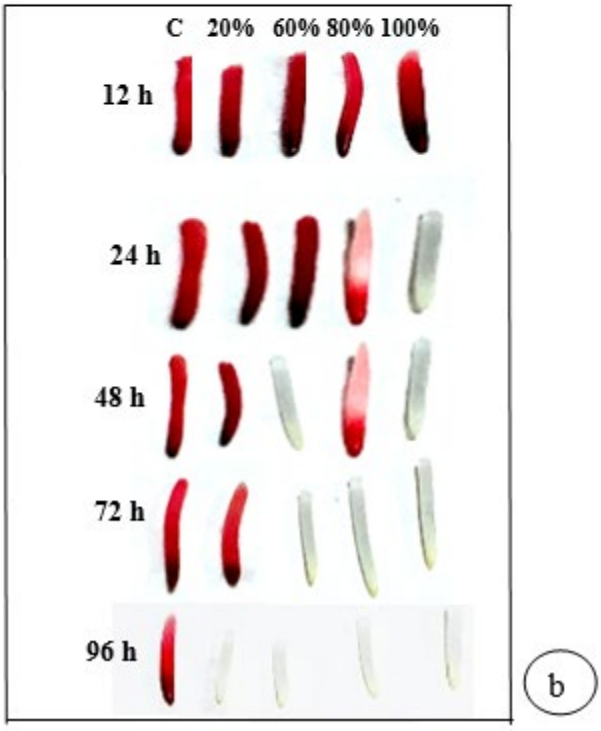


Fig. 5: (a) Surface view by Scanning electron microscopy of root tip of *A. cepa* treated with different concentration of PPMW along with control; (b) TTC test in *A. cep*a root at different concentration of PPMW and different time incubation.

3.9. Cell inhibition and chromosomal aberration

The qualitative effect of PPMW on chromosomes at distinct phases of the cell cycle in untreated and treated root meristematic cells is shown in Fig.6. The genotoxic study showed a concentration-

dependent decrease (%) in the mitotic index (MI) of *A. cepa* root tip treated with PPMW 50% and 100% concentration (Table 3). The decrease and increase in MI are important indicators of the presence of mutagenic and cytotoxic compounds. The PPMW appeared to induced chromosome abnormalities in *A. cepa*. The normal chromosomes (control) were presented in Fig. 6 (a-d) and abnormalities in the treated samples were shown in Fig. 6 (e-p). Abnormalities include ring chromosome, breaks and gaps, vagrant chromosome, irregular single chromosome bridge at anaphase, chained anaphase showing pulverization, chromosome fragment at metaphase, lagging chromosome, laggard chromosome, sticky anaphase, scattered polyploidy cell, shrink chromosome, c-metaphase and sticky chromosome at anaphase. The existence of toxic chemicals in the PPMW might have disturbed the division, resulting in a relatively high number of aberrations. The mitotic inhibition of neat (100%) PPMW is around 96.15% to total inhibition, while the 50 % PPMW showed 88.36% inhibition (Table3). Chromosomal aberration could be related to the polycyclic aromatic hydrocarbons (PAHs) present in the PPMW; PAHs with 5 or more rings could be mutagenic and carcinogenic (Aina et al., 2006). Some organic compounds detected from pulp and paper mill effluent in a separate study also showed chromosomal damage and chromosomal aberration in *A. cepa* (Yadav and Chandra, 2018; Haq et al. 2017). The frequencies of the cell with laggard and stickiness chromosome increased with increase PPMW concentration. These results are consistent with the data of the previous report of androgenic, mutagenic compounds in other industrial waste (Chandra and Kumar, 2017). The organic pollutants disturbed the balance in the number of histones or other proteins that are responsible for controlling the proper structure of nuclear chromatin, single-strand breaks in cellular DNA as well as a chromosomal aberration and DNA-protein cross-links lead to abnormalities during cell division (Chandra et al. 2017). Chromosome stickiness reflects a highly toxic effect, probably

leading to cell death (Leme et al. 2009). The chromosome bridge originated from the dicentric chromosome at anaphase in PPMW treated *A. cepa* could be attributed to the miss-repair of DNA, telomere end fusions, or even from chromosome adherence. The enhanced stickiness also contributes to the formation of Chromosome Bridge. Similar chromosomal aberration in *A. cepa* caused by municipal water in the constructed wetland was reported by (Firbas and Amon. 2013).

3.10. Observation of metal accumulation in root tissue of A. cepa

The accumulation of organometallic compounds in a different part of the root cells of *A. cepa* after exposure to PPMW was presented in Fig.6.The TEM analysis of *A. cepa* root tissue showed the damage of nuclear membrane, mitochondria, vacuole, change the nuclear shape and cell membrane deposition of metals granules in cell cytoplasm were observed in Fig.6a-d. Similar accumulation and damage to cell organelles in *A. cepa* after exposure to ZnO nanoparticles was observed by (Ahmed et al. 2015). The concentration of heavy metals in PPMW was present in very high value (Table 1). The deformities of cell shape indicated the disturbance in ligninfications of the cell wall, probably resulting from the accumulation of metals in *A. cepa* that induced peroxidase activity during plant growth. The cells were irregular and damaged due to the accumulation of ions and metals in their cell membrane (Chandra et al., 2010). Moreover, the mitochondrial cells appeared to shrink, and intracellular space in cytoplasm formed between the cells (Fig.6d). The high concentration of different metals in PPMW likely to have overwhelmed the stress tolerance in *A. cepa* and resulted in nucleus damage.

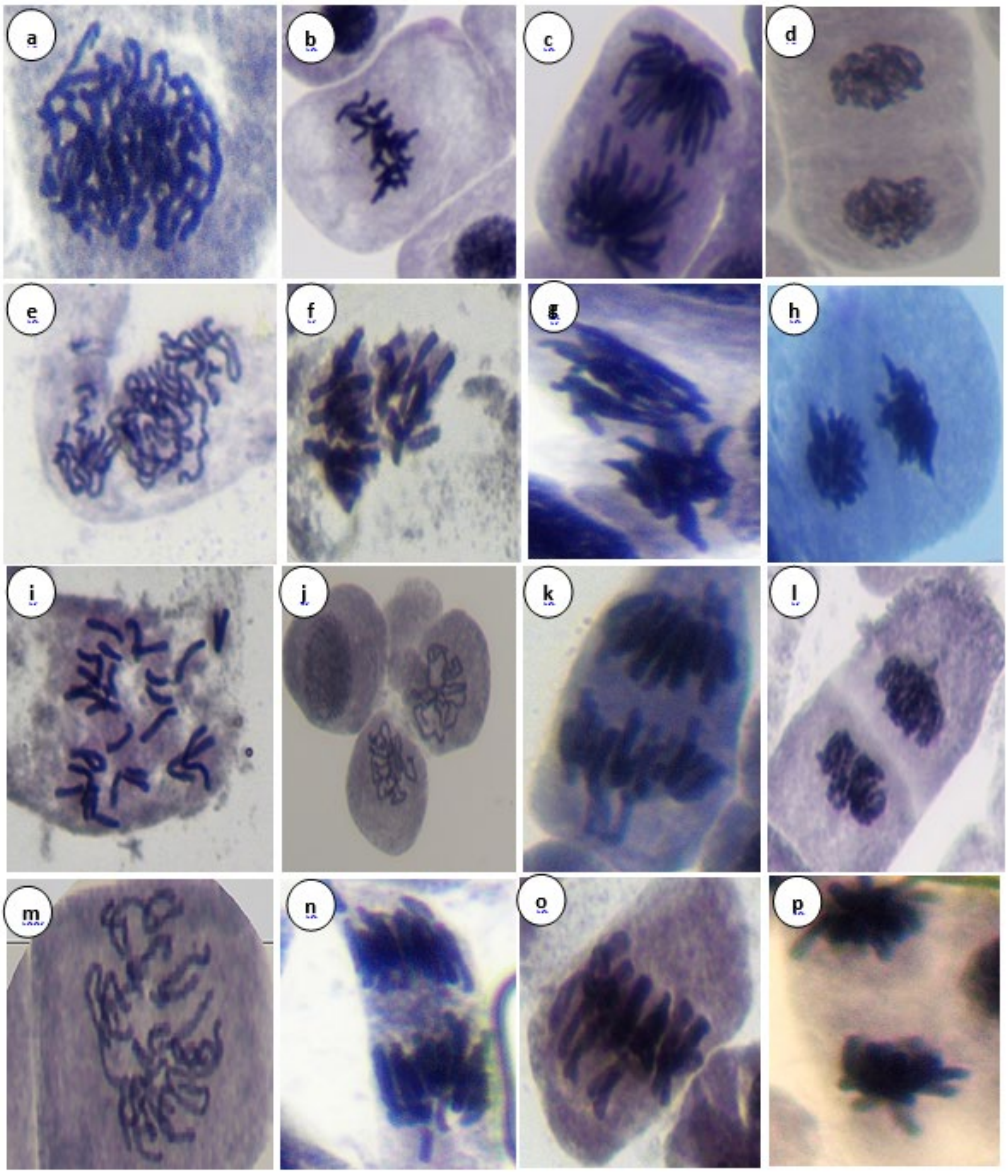


Fig. 6: Microscopic analyses of chromosomal damage in *A. cepa* root meristem cells induced by 100% and 50% of PPMW in *A. cepa* root meristem cells at different cell division stages of chromosome damage (a) Normal prophase; (b) Normal metaphase; (c) Normal anaphase; (d) normal telophase; (e) Metaphase with ring chromosome, breaks and gaps; (f) vagrant chromosome; (g) Irregular single chromosome bridge at anaphase; (h) Chained anaphase showing pulverization; (i) chromosome fragment at metaphase; (j) lagging chromosome; (k) laggard chromosome; (l) Sticky anaphase; (m) Scattered polyploidy cell; (n) shrink chromosome; (o) c-metaphase; and (p) sticky chromosome at anaphase.

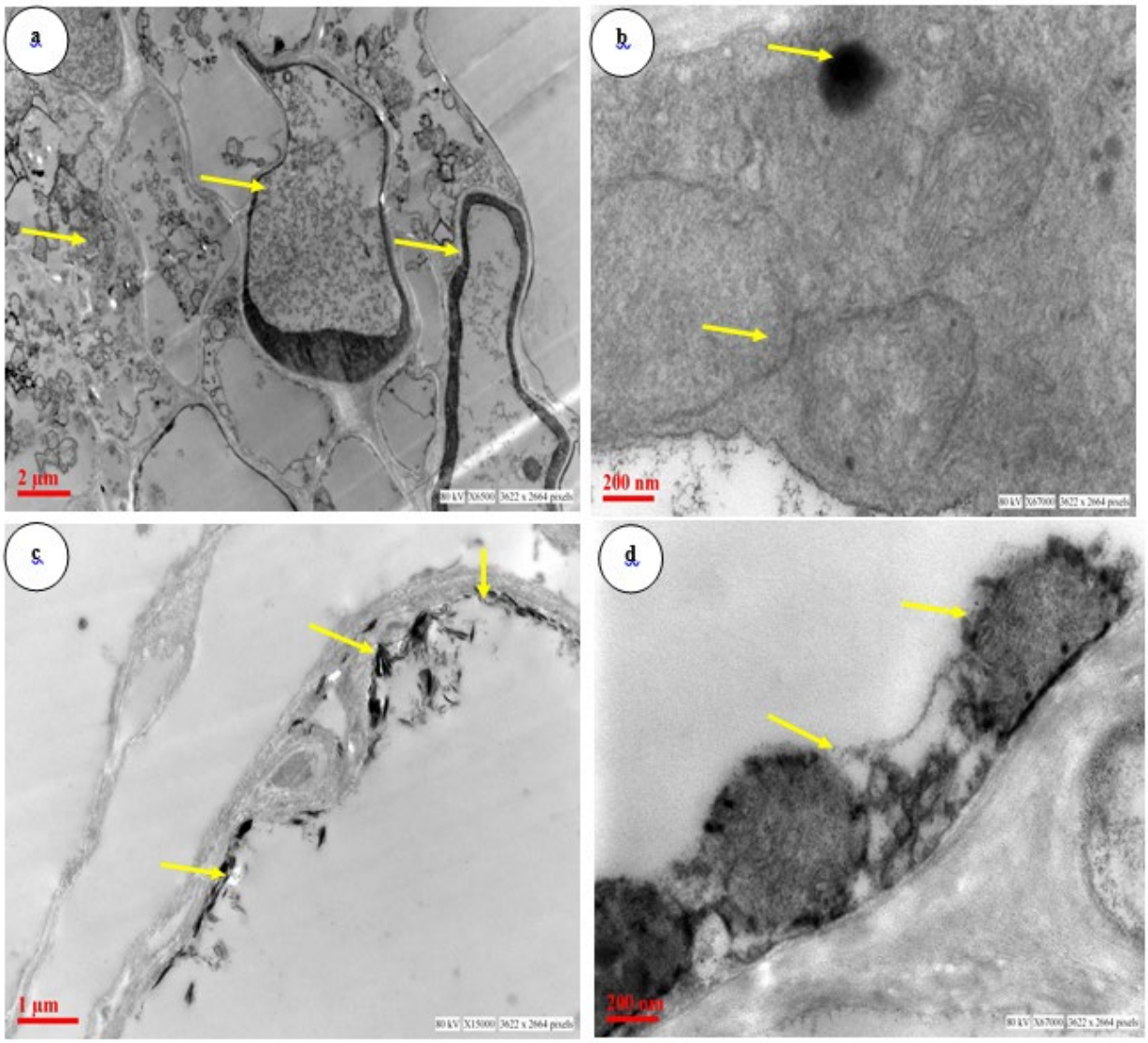


Fig.7: TEM analysis showing internal and chromosomal damage in *A. cepa* root cells upon exposure with PPMW. (a-d) Magnified view of roots after treatment showing the influx of metals in cytoplasm, mitochondria, and vacuoles. Arrow showing attachment of metals onto the cell membrane and analysis was performed at 1, 2, and 200 nm.

4. Conclusion

This study concluded that even after the secondary treatment PPMW contained various organometallic pollutants along with a high concentration of other physico-chemical parameters

that caused phytotoxicity, chromosomal break, cytotoxicity and oxidative damage of DNA in *A. cepa*. The predominant detected pollutants were fatty acids such as nonacosane, heptacosane, Hexadecanoic acid, stearic acid, 3-(octadecyloxy) propyl ester (CAS), Octadecane, and octadecanoic, etc. Other residual organic pollutants were also detected, including (4-chlorophenyl)-2,5,5-triphenyl-5,8-6H-azeto[1,2a] [1,3] thiazole [4,5-d] pyrimidine; 6-benzoamido-3-[2-[1-(phenylmethyl)-4-piperidinyl] ethyl]-1,2-benzisoxazole; Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) and ergostan-6-one, that caused direct toxicity to the aquatic ecosystem. The toxicity assessment via *P. Mungo* seed germination and *A. cepa* to toxicity showed inhibition of α-amylase and reduction in a mitotic index. The selected model plants are important ecosystem and environmental pollution indicator and the observed genotoxicity, and cytotoxicity of PPMW indicate there is an urgent need for the development and optimization of the tertiary stage treatment process before PPMW could be discharged safely into the environment.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Afroz, Z., & Singh, A. (2014). Impact of pulp and paper mill effluent on water quality of river Aami and its effect on aquatic life (fish). *Global J. Pharmacol*, *8*(2), 140-149.

Afshan, S., Ali, S., Ameen, U. S., Farid, M., Bharwana, S. A., Hannan, F., & Ahmad, R. (2014). Effect of different heavy metal pollution on fish. *Research journal of chemical and environmental sciences*, *2*(1), 74-79.

Ahmed, E. M. (2015). Hydrogel: Preparation, characterization, and applications: A review. *Journal of advanced research*, *6*(2), 105-121.

Ahmad, I., Maria, V. L., Oliveira, M., Pacheco, M., & Santos, M. A. (2006). Oxidative stress and genotoxic effects in gill and kidney of Anguilla anguilla L. exposed to chromium with or without pre-exposure to β-naphthoflavone. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *608*(1), 16-28.

Ahmad, I., Maria, V. L., Oliveira, M., Pacheco, M., & Santos, M. A. (2006). Oxidative stress and genotoxic effects in gill and kidney of Anguilla anguilla L. exposed to chromium with or without pre-exposure to β-naphthoflavone. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *608*(1), 16-28.

Aina, R., Palin, L., & Citterio, S. (2006). Molecular evidence for benzo [a] pyrene and naphthalene genotoxicity in Trifolium repens L. *Chemosphere*, *65*(4), 666-673.

Ali, M., & Sreekrishnan, T. R. (2001). Aquatic toxicity from pulp and paper mill effluents: a review. *Advances in environmental research*, *5*(2), 175-196.

Central pulp paper research Institute (CPPRI) Annual report (2016-17) Saharanpur (U.P.) India.

APHA (American Public Health Association): Standard Method for Examination of Water and Wastewater, 22nd ed. Washington, DC. (2012).

Batista, A. H., Melo, V. F., Gilkes, R., & Roberts, M. (2018). Identification of heavy metals in crystals of sand and silt fractions of soils by scanning electron microscopy (SEM-EDS/WD-EPMA). *Revista Brasileira de Ciência do Solo*, *42*.

Bharagava, R. N., & Chandra, R. (2010). Effect of bacteria treated and untreated post-methanated distillery effluent (PMDE) on seed germination, seedling growth, and amylase activity in Phaseolus mungo L. *Journal of hazardous materials*, *180*(1-3), 730-734.

Carranzo, I. V. (2012, July). Standard Methods for examination of water and wastewater. In *Anales De Hidrología Médica* (Vol. 5, No. 2, p. 185). Universidad Complutense de Madrid.

Chandra, R., & Abhishek, A. (2011). Bacterial decolorization of black liquor in axenic and mixed condition and characterization of metabolites. *Biodegradation*, *22*(3), 603-611.

Chandra, R., & Kumar, V. (2017). Detection of androgenic-mutagenic compounds and potential autochthonous bacterial communities during in situ bioremediation of post-methanated distillery sludge. *Frontiers in microbiology*, *8*, 887.

Chandra, R., & Singh, R. (2012). Decolourisation and detoxification of rayon grade pulp paper mill effluent by mixed bacterial culture isolated from pulp paper mill effluent polluted site. *Biochemical Engineering Journal*, *61*, 49-58.

Chandra, R., & Yadav, S. (2010). The potential of Typha angustifolia for phytoremediation of heavy metals from an aqueous solution of phenol and melanoidin. *Ecological Engineering*, *36*(10), 1277-1284.

Chandra, R., Raj, A., Yadav, S., & Patel, D. K. (2009). Reduction of pollutants in pulp paper mill effluent treated by PCP-degrading bacterial strains. *Environmental monitoring and assessment*, *155*(1-4), 1.

Chandra, R., Sharma, P., Yadav, S., & Tripathi, S. (2018). Biodegradation of endocrine-disrupting chemicals and residual organic pollutants of pulp and paper mill effluent by biostimulation. *Frontiers in microbiology*, *9*, 960.

Chandra, R., Singh, R., & Yadav, S. (2012). Effect of bacterial inoculums ratio in mixed culture for decolorization and detoxification of pulp paper mill effluent. *Journal of Chemical Technology & Biotechnology*, *87*(3), 436-444.

Chandra, R., Singh, S., & Raj, A. (2006). Seasonal bacteriological analysis of Gola river water contaminated with pulp paper mill waste in Uttaranchal, India. *Environmental monitoring and assessment*, *118*(1-3), 393-406.

Chandra, R., Yadav, S., & Yadav, S. (2017). Phytoextraction potential of heavy metals by native wetland plants growing on chlorolignin containing sludge of pulp and paper industry. *Ecological Engineering*, *98*, 134-145.

Chiang, G., Barra, R., Díaz-Jaramillo, M., Rivas, M., Bahamonde, P., & Munkittrick, K. R. (2015). Estrogenicity and intersex in juvenile rainbow trout (Oncorhynchus mykiss) exposed to Pine/Eucalyptus pulp and paper production effluent in Chile. *Aquatic Toxicology*, *164*, 126-134.

Chowdhury, U. K., Biswas, B. K., Chowdhury, T. R., Samanta, G., Mandal, B. K., Basu, G. C., ... & Roy, S. (2000). Groundwater arsenic contamination in Bangladesh and West Bengal, India. *Environmental health perspectives*, *108*(5), 393-397.

de Sousa Andrade, L. N., De Lima, T. M., Curi, R., & de Lauro Castrucci, A. M. (2005). Toxicity of fatty acids on murine and human melanoma cell lines. *Toxicology in vitro*, *19*(4), 553-560.

Deepali, K. K., Gangwar, R., & Joshi, B. D. (2009). Comparative study of Physico-chemical properties of effluent from tannery industries. *Indian Journal of Environmental Sciences*, *3*(2), 49-152.

EPA (2012) seedling emergence and seedling growth emergence and growth test PPT-21.

Farag, A. M., May, T., Marty, G. D., Easton, M., Harper, D. D., Little, E. E., & Cleveland, L. (2006). The effect of chronic chromium exposure on the health of Chinook salmon (Oncorhynchus tshawytscha). *Aquatic toxicology*, *76*(3-4), 246-257.

Fendri, I., Ben Saad, R., Khemakhem, B., Ben Halima, N., Gdoura, R., & Abdelkafi, S. (2013). Effect of treated and untreated domestic wastewater on seed germination, seedling growth and amylase and lipase activities in Avena sativa L. *Journal of the Science of Food and Agriculture*, *93*(7), 1568-1574.

Firbas, P., & Amon, T. (2013). Allium chromosome aberration test for evaluation effect of cleaning municipal water with Constructed Wetland (CW) in Sveti Tomaž, Slovenia. *Journal of Bioremediation and Biodegradation*, *4*(4), 189.

Firbas, P., & Amon, T. (2014). Chromosome damage studies in the onion plant Allium cepa L. *Caryologia*, *67*(1), 25-35.

Gauthier, F., & Archibald, F. (2001). The ecology of "fecal indicator" bacteria commonly found in pulp and paper mill water systems. *Water Research*, *35*(9), 2207-2218.

González, T., Terrón, M. C., Yagüe, S., Zapico, E., Galletti, G. C., & González, A. E. (2000). Pyrolysis/gas chromatography/mass spectrometry monitoring of fungal-biotreated distillery wastewater using Trametes sp. I-62 (CECT 20197). *Rapid communications in mass spectrometry, 14,* 1417-1424.

Gustavo, C., Ricardo, B., Mauricio, D., Meyling, R., Paulina, B., Kelly, R., Munkittrick, (2015)

Estrogencity, and intersex in juvenile rainbow trout (*Oncorhynchusmy kiss*) exposed to Pine/ Eucalyptus pulp and paper production effluent in Chile. *Aquatic Toxicology*.164, 126–134.

Hankin, L., & Kolattukudy, P. E. (1968). Metabolism of a plant wax paraffin (n-nonacosane) by a soil bacterium (Micrococcus cerificans). *Microbiology*, *51*(3), 457-463.

Haq, I., Kumar, S., Raj, A., Lohani, M., & Satyanarayana, G. N. V. (2017). Genotoxicity assessment of pulp and paper mill effluent before and after bacterial degradation using the *Allium cepa* test. *Chemosphere*, *169*, 642-650.

Hou, L. P., Yang, Y., Shu, H., Ying, G. G., Zhao, J. L., Fang, G. Z., ... & Cheng, X. M. (2018). Masculinization and reproductive effects in western mosquitofish (Gambusia affinis) after long-term exposure to androstenedione. *Ecotoxicology and environmental safety*, *147*, 509-515.

Huang, M., Qi, F., Shi, X., & Hu, H. (2015). COD fractionation and toxicity of pulp and paper mill wastewaters in a tertiary process. *Desalination and Water Treatment*, *56*(3), 615-621.

Ilić, S. Z., Mirecki, N., Trajković, R., Kapoulas, N., Milenković, L., & Šunić, L. (2015). Effect of Pb on germination of different seed and his translocation in bean seed tissues during sprouting. *Fresenius Environ Bullutin*, *24*(2A), 670-675.

Iqbal, H. M. N., Kyazze, G., & Keshavarz, T. (2013). Advances in the valorization of lignocellulosic materials by biotechnology: an overview. *BioResources*, *8*(2), 3157-3176.

Ivanov, V. B., Bystrova, E. I., & Seregin, I. V. (2003). Comparative impacts of heavy metals on root growth as related to their specificity and selectivity. *Russian Journal of Plant Physiology*, *50*(3), 398-406.

Kågedal, K., Bironaite, D., & Öllinger, K. (1999). Anthraquinone cytotoxicity and apoptosis in primary cultures of rat hepatocytes. *Free radical research*, *31*(5), 419-428.

Karrasch, B., Parra, O., Cid, H., Mehrens, M., Pacheco, P., Urrutia, R., & Zaror, C. (2006). Effects of pulp and paper mill effluents on the microplankton and microbial self-purification capabilities of the Biobio River, Chile. *Science of the Total Environment*, *359*(1-3), 194-208.

Kaushik, G., Gopal, M., & Thakur, I. S. (2010). Evaluation of performance and community dynamics of microorganisms during treatment of distillery spent wash in a three-stage bioreactor. *Bioresource Technology*, *101*(12), 4296-4305.

Khan, S. R., Finlayson, B., & Hackett, R. (1984). Renal papillary changes in patient with calcium oxalate lithiasis. *Urology*, *23*(2), 194-199.

Kim, S., Thiessen, P. A., Bolton, E. E., Chen, J., Fu, G., Gindulyte, A., ... & Wang, J. (2016). PubChem substance and compound databases. *Nucleic acids research*, *44*(D1), D1202-D1213.

Kumar, V., Chopra, A. K., Kumar, S., Singh, J., & Thakur, R. K. (2015). Effects of pulp and paper mill effluent disposal on soil characteristics in the vicinity of Uttaranchal Pulp and Paper Mill, Haridwar (Uttarakhand), India. *International Journal of Agriculture Science and Research*, *4*, 117-125.

Lacorte, S., Latorre, A., Barcelo, D., Rigol, A., Malmqvist, A., & Welander, T. (2003). Organic compounds in paper-mill process waters and effluents. *TrAC Trends in Analytical Chemistry*, *22*(10), 725-737.

Ladwani, K. D., Ladwani, K. D., Ramteke, D. S., & Deo, S. (2016). Detection and Identification of Organic Compounds in Wastewater of Final Effluent Treatment Plant by FTIR and GC-MS. *Journal of Advanced Chemical Sciences*, 246-247.

Leach, J. M., & Thakore, A. N. (1978, January). Compounds toxic to fish in pulp mill waste streams. In *Eighth International Conference on Water Pollution Research* (pp. 787-798). Pergamon.

Leme, D. M., & Marin-Morales, M. A. (2009). Allium cepa test in environmental monitoring: a review on its application. *Mutation Research/Reviews in Mutation Research*, *682*(1), 71-81.

Lindholm-Lehto, P. C., Knuutinen, J. S., Ahkola, H. S., & Herve, S. H. (2015). Refractory organic pollutants and toxicity in pulp and paper mill wastewaters. *Environmental Science and Pollution Research*, *22*(9), 6473-6499.

Liu, X., Zhu, H., Qin, C., Zhou, J., Zhao, J. R., & Wang, S. (2013). Adsorption of heavy metal ion from aqueous single metal solution by aminated epoxy-lignin. *BioResources*, *8*(2), 2257-2269.

Madan, S., Sachan, P., & Singh, U. (2018). A review on bioremediation of pulp and paper mill effluent â€“An alternative to conventional remedial technologies. *Journal of Applied and Natural Science*, *10*(1), 367-374.

Maria, S., Sepulveda, William, E., Johnson, John, C., Higman, Nancy, D., Denslow, Trenton, R., Schoeb, Timothy, S., Gross, 2002. Evaluation of biomarkers of reproductive function and potential contaminant effects in Florida largemouth bass (*Micropterus salmoides floridanus*) sampled from the St. Johns River. Sci. Total Environ. 289, 133–144.

McMartin, D. W., Peru, K. M., Headley, J. V., Winkler, M., & Gillies, J. A. (2002). Evaluation of liquid chromatography–negative ion electro spray mass spectrometry for the determination of selected resin acids in river water. *Journal of Chromatography A*, *952*(1-2), 289-293.

Morrison, W. H., & Akin, D. E. (2001). Chemical composition of components comprising bast tissue in flax. *Journal of agricultural and food chemistry*, *49*(5), 2333-2338.

Müller, J. B., Melegari, S. P., Perreault, F., & Matias, W. G. (2019). Comparative assessment of acute and chronic ecotoxicity of water soluble fractions of diesel and biodiesel on Daphnia magna and Aliivibrio fischeri. *Chemosphere*, *221*, 640-646.

Nefic, H., Musanovic, J., Metovic, A., & Kurteshi, K. (2013). Chromosomal and nuclear alterations in root tip cells of Allium cepa L. induced by alprazolam. *Medical archives*, *67*(6), 388.

Nurmesniemi, H., Pöykiö, R., Perämäki, P., & Kuokkanen, T. (2005). The use of a sequential leaching procedure for heavy metal fractionation in green liquor dregs from a causticizing process at a pulp mill. *Chemosphere*, *61*(10), 1475-1484.

OECD Guideline for testing of chemicals (2006). Terrestrial plant tests: 208: Seedling emergence and seedling growth test. 1-21.

Ojunga, S., Masese, F. O., Manyala, J. O., Etiégni, L., Onkware, A. O., Senelwa, K., ... & Omutange, E. S. (2010). Impact of a kraft pulp and paper mill effluent on phytoplankton and macroinvertebrates in River Nzoia, Kenya. *Water Quality Research Journal*, *45*(2), 235-250.

Ott, L. (1984). *An Introduction to Statistical Methods and Data Analysis*. Duxbury.

Pearl, I. A., & Benson, H. K. (1940). The determination of lignin in sulfite pulping liquor. *Paper Trade Journal*, *111*, 35-36.

Peng, W. X., & Wu, Y. Q. (2008). Py-GC/MS analysis on benzene/ethanol extractives of Eucalyptus camaldulensis wood meal in pulping wastewater from South China. In *Proceedings of 2008 conference on the regional economy and sustainable development*.

Pokhrel, D., & Viraraghavan, T. (2004). Treatment of pulp and paper mill wastewater—a review. *Science of the total environment*, *333*(1-3), 37-58.

Reddy, P. M., & Rao, N. S. (2001). Effects of Industrial Effluents on the Groundwater Regime in Visakhapatnam. *Pollution Research*, *20*(3), 383-386.

Réveillé, V., Mansuy, L., Jardé, É., & Garnier-Sillam, É. (2003). Characterization of sewage sludge-derived organic matter: lipids and humic acids. *Organic Geochemistry*, *34*(4), 615-627.

Savant, D. V., Abdul-Rahman, R., & Ranade, D. R. (2006). Anaerobic degradation of adsorbable organic halides (AOX) from pulp and paper industry wastewater. *Bioresource Technology*, *97*(9), 1092-1104.

Shaymurat, T., Gu, J., Xu, C., Yang, Z., Zhao, Q., Liu, Y., & Liu, Y. (2012). Phytotoxic and genotoxic effects of ZnO nanoparticles on garlic (Allium sativum L.): a morphological study. *Nanotoxicology*, *6*(3), 241-248.

Singh, A. K., & Chandra, R. (2019). Pollutants released from the pulp paper industry: Aquatic toxicity and their health hazards. *Aquatic Toxicology*.

Singh, V. K., Kavita, K., Prabhakaran, R., & Jha, B. (2013). Cis-9-octadecenoic acid from the rhizospheric bacterium Stenotrophomonas maltophilia BJ01 shows quorum quenching and anti-biofilm activities. *Biofouling*, *29*(7), 855-867.

Slavin, Y. N., Asnis, J., Häfeli, U. O., & Bach, H. (2017). Metal nanoparticles: understanding the mechanisms behind the antibacterial activity. *Journal of nanobiotechnology*, *15*(1), 65.

Smith, A. H., Lingas, E. O., & Rahman, M. (2000). Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bulletin of the World Health Organization*, *78*, 1093-1103.

Suntio, L. R., Shiu, W. Y., & Mackay, D. (1988). A review of the nature and properties of chemicals present in pulp mill effluents. *Chemosphere*, *17*(7), 1249-1290.

Taylor, C. D., Smith, S. O., & Gagosian, R. B. (1981). Use of microbial enrichment for the study of the anaerobic degradation of cholesterol. *Geochimica et Cosmochimica Acta*, *45*(11), 2161-2168.

USEPA (2002) the environment protection rules, 3A, Schedule-II, III. U.S. Environmental Protection Agency, Office of research and development, Cincinnati.

USEPA, U.S. Environmental Protection Agency (2012). Endocrine Disruptor Screening Program, Universe of Chemicals for Potential Endocrine Disruptor Screening and Testing, Jointly developed by the Office of Chemical Safety & Pollution Prevention, the Office of Water and the Office of Research and Development, 1-176.

Verma, K., Shekhawat, G. S., Sharma, A., Mehta, S. K., & Sharma, V. (2008). Cadmium-induced oxidative stress and changes in soluble and ionically bound cell wall peroxidase activities in roots of seedling and 3–4 leaf stage plants of Brassica juncea (L.) czern. *Plant Cell Reports*, *27*(7), 1261-1269.

WONG, M. H., & Bradshaw, A. D. (1982). A comparison of the toxicity of heavy metals, using root elongation of ryegrass, Lolium perenne. *New Phytologist*, *91*(2), 255-261.

Wu, G., Guan, Y., & Zhan, X. (2008). Effect of salinity on the activity, settling and microbial community of activated sludge in sequencing batch reactors treating synthetic saline wastewater. *Water Science and Technology*, *58*(2), 351-358.

Yadav, S., & Chandra, R. (2018). Detection and assessment of the phytotoxicity of residual organic pollutants in sediment contaminated with pulp and paper mill effluent. *Environmental monitoring and assessment*, *190*(10), 581.