Characterization of persistent organic pollutants and culturable and non-culturable bacterial communities in pulp and paper sludge after secondary treatment

Sonam Tripathi [a], Sangeeta Yadav [a,*], Diane Purchase [b], Kaman Singh [c], Hind A. AL-Shwaiman [d], Ram Chandra [a,*]

[a] *Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow, 226025, (U.P.), India*

[b] *Department of Natural Sciences, Faculty of Science and Technology, Middlesex University, The Burroughs, London NW4 4BT, UK*

[c] *Department of Chemistry, School of Physical & Decision Sciences, Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow, 226025 (U.P.), India* [d] *Department of Botany and Microbiology, College of Science, King Saud University, P.O 2455, Riyadh, 11451, Saudi Arabia*

*Corresponding author. *E-mail addresses:* dr.sangeeta.bbau@gmail.com (S. Yadav), prof.chandrabbau@gmail.com (R. Chandra).

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Highlights

- Persistent Organic Pollutants (POPs) were major pollutants in pulp-paper sludge.
- Six culturable and 40 viable but non-culturable bacterial strains were detected.
- The resistance to POPs by the culturable bacteria have not been previously reported.
- The remaining strains survived by entering viable but non-culturable state.
- The non-culturable bacterial strains has higher species richness and diversity.

Abbreviations

PPS	Pulp-paper sludge after secondary treatment
POPs	Persistent organic pollutants EC Electrical conductivity
L-MSM	lignin amended mineral salt medium
HPLC	High performance liquid chromatography DCM Dichloromethane
PAC	Polycyclic Aromatic Compounds
N	Normality

Abstract

Due to the presence of various organic contaminants, improper disposal of pulp-paper wastewater poses harm to the environment and human health. In this work, pulp-paper sludge (PPS) after secondary treatment were collected from M/s Century Pulp-paper Mills in India, the chemical nature of the organic pollutants was determined after solvent extraction. All the isolates were able to produce lipase (6.34–3.93 U ml^{-1}) which could account for the different fatty acids detected in the PPS. The dominant strains were in the classes of α and γ Proteobacteria followed by Firmicutes. The Shannon-Weiner diversity indexes for phylotype richness for the culturable and non-culturable bacterial community were 2.01 and 3.01, respectively, indicating the non- culturable bacterial strains has higher species richness and diversity compared to the culturable bacterial strains. However, the culturable strains had higher species evenness (0.94 vs 0.90). Results suggested only a few isolated strains were resistant to the POPs in the PPS, whereas non-cultural bacteria survived by entering viable but non-cultural state. The isolated strains (Brevundimonas diminuta, Aeromonas punctata, Enterobacter hormaechei, *Citrobacter braakii, Bacillus pumilus* and *Brevundimonas terrae*) are known for their multidrug resistance but their tolerance to POPs have not previously been reported and deserved further investigation. The findings of this research established the presence of POPs which influence the microbial population. Tertiary treatment is recommended prior to the safe disposal of pulp paper mill waste into the environment.

Keywords: Culturable and non-culturable bacterial stains; GC-MS; Lipase; Persistent organic pollutants; Pulp-paper mills sludge

1. Introduction

Persistent organic pollutants (POPs) are compounds resistant to chemical/biological breakdown and mobile in the environment. As they can bioaccumulate in human and animal tissues, even when they are present in extremely low concentrations in the environment, they can adversely affect the biota. POPs have also been proven to be genotoxic (or DNA damaging), carcinogenic and endocrine disrupting (Yadav and Chandra, 2018; Tripathi et al., 2021a). POPs present in industrial waste could cause chronic toxic effects and endocrine disruptions. As a result, USEPA considers POPs to be priority pollutants, and their discharge is strictly banned (Boguniewicz and Kłosok, 2020).

Pulp-paper mills are substantial sources of POPs where they are generated from the raw materials and their constituents during pulping. Soft and hard wood and non-wood fibres are used in pulp-paper mills as raw materials. Hence, natural wood extractives (such as tannins, resin acids and lignin) and xenobiotics compounds generated during pulping process of paper production (such as chlorinated lignin, phenols, dioxins and furans) are readily detected in pulp-paper wastewaters (Vidal et al., 2021). Therefore, pulp-paper mills are one of the most environmentally destructive sectors, discharging a wide range of toxic pollutants into the environment. This is exacerbated by the sheer scale of the discharge; the pulp-paper industry releases 170 million tonnes of hazardous contaminants into the environment each year worldwide (Haile et al., 2021). Furthermore, several researchers reported the inadequate secondary treatment failing to reduce toxic pollutants that are endocrine disrupting, carcinogenic and mutagenic for aquatic communities to a recommended level (Selvi et al., 2019; Kim et al., 2020). Pulp-paper mills discharge has also been described as a source of several pathogenic bacteria including coliform bacteria (Sahu et al., 2018; Schneider et al., 2020). Treated wastewater has been a major source of reused water for irrigation in India; many organic pollutants can enter the food chain via consumption of contaminated crops, fishes and animals (Lood et al., 2017; Chandra et al., 2011). Due to the different operation parameters and treatment efficiencies in the industry, comprehensive knowledge of POPs present in pulp-paper mills discharged and sludge are difficult to ascertain. However, several studies have found many chlorinated compounds, for example, chlorinated phenolics (di-chloroguicol, tri-chloroguicol, tetra-chloroguicol), di-chlorophenol, tri-chlorophenol, tetra-chlorophenol, pentachlorophenol, chlorinated resin acids and lignosulphonic acids in the effluent (Mukherjee et al., 2017; Kachienga et al., 2018). These refractory chloro-organic pollutants found in discharged of pulp-paper mills are recalcitrant, persist in the environment for a long time and have the capacity to travel far across the ecosystem, eventually accumulating in organisms' fatty tissues. Moreover, several anaerobic microbes have capacity to methylate the chlorinated pollutants which further increase the toxicity and lipophilicity of the pollutants (Rosso et al., 2018). As a result of the insufficient secondary treatment, effluent from pulp and paper mills could contain significant concentrations of persistent pollutants that impaction microbial activities in the environment (Sharma et al., 2021a; Tripathi et al., 2021b). Hence, this research aims to identify the POPs present in the pulp-paper sludge (PPS) discharged after the secondary treatment and their effect on microbial community present in

the PPS. The study aims to provide strong evidence on the efficiency of the secondary treatment and the impact on the microbial diversity in the immediate environment. The new knowledge on the POPs and bacterial biodiversity in pulp-paper mills sludge will help to develop a better management and treatment regime for the industry in India and facilitate sustainable ecological restoration.

2. Material and methods

2.1. Collection of sludge from pulp-paper mills after secondary treatment (PPS)

M/s Century Pulp Paper Mill, Uttarakhand, India (located at 29°03′45.4″N 79°31′25.9″E provided the PPS for this investigation. The 20 kg PPS samples in triplicate were randomly collected and brought to laboratory and store at field humidity in polyethylene bags until processing. Organic pollutants, physico-chemical analysis and identification of bacterial communities were done in each sample.

2.2. Physico-chemical analysis of the PPS

All physico-chemical analysis, i.e., electrical conductivity (EC), phosphate, sulfate, total nitrogen, total organic matter, Na^+, K^+ and Cl^-, were completed within 24 h of sample collection. All the physico- chemical pollution parameters were analysed by using the methods described in APHA (2017). The samples were prepared by combining 50 g PPS with 500 ml of autoclaved distilled water. The pH and electrical conductivity were measured using respective electrode in an Orion ion meter (Model-960). Vanadomolybdo-phosphoric acid colorimetric and $BaCl_2$ precipitation techniques were used for analysis of phosphate and sulfate concentrations, respectively in the PPS samples. TOC-Vcsnanalyzer (Shimadzu, Japan) was used for total nitrogen estimation. For metal detection, i.e., Cr, Cu, Fe, Mn, Ni, Pb and Zn, the samples were digested using the standard process described in the EPA (1996) and the concentration was measured using an inductively coupled plasma spectrophotometer (IRIS Intrepid II XDL, Thermo Electron, USA). All the procedures were repeated three times as well as statistical analyses were performed by Graph Pad Software (Graph Pad Software, San Diego, Calif) for significance of data (Ott et al., 1984).

2.3. Identification and characterization of organic contaminants found in the PPS

2.3.1. Extraction of POPs from the PPS

A microwave assisted Soxhlet was used to extract a solvent from an aliquot of the dried and sieved PPS. Several organic solvents were used as extracting solvents, and ethyl acetate and dichloromethane (DCM) were determined to be the best. The operational processes were as follows: sludge samples weighing 50 g were diluted in 500 ml distilled water and shaken for 24 h at 100 rpm. The suspended particles were removed by centrifugation of solution at 3000×*g* for 20 min. The resulting supernatant was maintained at pH 2.0 with the help of 1 M HCl, organic contaminants were extracted with equal volume of ethyl acetate and DCM in a 500 ml separating funnel after intermittent shaking (Yadav and Chandra, 2018). The upper layer contained organic contaminants was collected and dried under vacuum at 40 ˚C. The dried residue was dissolved in 1 ml HPLC grade acetonitrile then filtered using a 0.22 m syringe filter. The filtered extracts were kept at 5 ˚C until further analysis.

2.3.2. Gas chromatography-mass spectrophotometry (GC–MS) analysis

GC-MS has been used utilised successfully to identify and quantify the pollutants present in PPS (Yadav and Chandra, 2015). Silyl derivatization is an effective method for identifying existing non-volatile chemicals. The combination of gas chromatography (GC) with mass spectrometry (GC-MS) increases the sensitivity and selectivity of the analysis (Ignatet al, 2011). Prior to GC-MS analysis, the sample were treated with 100 μl dioxane and 10 μl pyridine before silylation. Siylation was carried out with 50 l trimethyl silyl [BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and trimethyl chlorosilane. The solutions were heated at temperature 60 ˚C for 15 min with intermittent shaking to dissolve the residues. A prepared sample (1 μl) was loaded into a PE auto system XL gas chromatograph (PerkinElmer, UK) attached with a Turbo-mass spectrometric mass selective detector. PE-5MS capillary column was the analytical column attached to the system (20 m 0.18 mm internal diameter, 0.18 mm film thickness). Helium gas was employed as the carrier gas during GC-MS analysis and maintained the flow rate 1 ml min^{-1}. The column temperature was set at 50 ˚C for 5 min; 50–300 ˚C for 10 ˚C min^{-1} with a hold time of 5 min. The temperatures of the transfer line and the ion source were kept constant at 200 and 250 ˚C, respectively. A solvent delay of 3.0 min was used. The electron ionization (EI) mass spectra were obtained in full-scan mode at 70 eV in

the range of 30–550 (*m*/*z*). The organic pollutants were detected by comparing their mass spectra to the compounds present in NIST library that came with the equipment, simultaneously RT of detected compounds are also compared with actual organic compounds that were accessible.

2.4. Bacterial community analysis in the PPS

2.4.1. Culturable bacteria

For the isolation of culturable bacteria from the PPS, enrichment procedure was performed using lignin amended mineral salt medium (L- MSM) (Sharma et al., 2021b). Flasks were aerobically incubated at 30 ˚C in a temperature-controlled shaking incubator at 120 rpm in darkness for 7 d. The bacterial population that emerged in the enrichment culture of the non-sterile PPS is referred to as culturable bacteria in this study. One ml of this culture was erially diluted and 1 μl of this spread on L-MSM agar plates (HiMedia, India) and incubated at 30 ˚C. Single colonies with diverse morphologies were selected from each plate and purified by streaking them onto fresh L-MSM agar plates. Purified colonies were used for further investigation.

2.4.2. Identification of culturable bacteria by 16 S rRNA

For 16 S rRNA gene sequence analysis, total DNA was extracted from overnight culture from pure cells based on the alkaline lysis procedure.16 S rRNA was successfully amplified using the universal primers described by Narde et al. (2004). To increase the quantity of the desired product, PCR was performed: 35 cycles of denaturation (94 ˚C, 1 min), annealing (55 ˚C, 1.5min) and extension (72 ˚C, 1 min). Using a forward primer 5′-3’: AGAGTTTGATCMTGGCTCAG and reverse primer 5′-3’: TACGGYTACCTTACGACTT 1466 bp long nucleotide was successfully amplified. The bacteria were identified based on maximum similarity on BLAST and their sequences submitted tohttp://www.ncbi.nlm.nih.gov/BLAST.

2.4.3. Non-culturable bacterial communities in the PPS

DNA extraction kit purchased from Bangalore Genei, India (GeNei™ Genomic DNA Extraction Kit, Bangalore Genei, India) was used to obtain whole genomic DNA from the PPS. Consensus primers were used for amplification of 16 S rRNA gene during PCR analysis. The 25 μl of PCR reaction mixture contained 1 μl of template DNA, 2.5 μl of 10 x PCR buffer, 1 μl of 2.5 mM of

each dNTP, 1 μl of primer, and 3.0 U of XT5 DNA polymerase (Bangalore Genei, India). The DNA was amplified via 35 cycles of PCR: denaturation (94 °C, 30 s), annealing (53 °C, 45s) and elongation (72 °C, 1.3 min), with a 5 min beginning denaturation (94 °C, 10 min) and final extension phase at 72 °C. Agarose gels (1.2%) were used to separate the amplified products. The1.5 kb amplification product was gel-purified using a PCR- Clean-up kit (Bangalore Genei, India) and cloned into a TA cloning vector within the *Nco*I sites (Instant ligation kit, Bangalore Genei, India). The recombinant clones were initially screened by the release of the insert using *Noc*I restriction endonuclease. The insert was re-amplified using the consequence primers from the confirmed clones. The amplified product was subjected to PCR-RFLP using two different restriction enzymes (tetra cutters namely *Taq*I and *Sau3A*I). The polymorphic clones were purified and bidirectionally sequenced using two universal primers M13F (5′-GTAAAACGACGGCCAGT-3′) and M13R (5′-AACAGCTATGACCATG-3′). BLAST was used to determine the clones that corresponded to the sequencing data. The detected strains' accession numbers were placed in Gene Bank, and the accession numbers are accessible in the online supplement, which may be read at http://www.ncbi.nlm.nih.gov/.

2.5. Lipase activity detection and quantification

Bacterial strains were inoculated on agar plates containing Tween 20 and incubated at 35 °C to measure the lipase activity (El-Bestawy et al., 2005). Lipase activity was demonstrated by the production of white precipitation at the edge of the bacterial colony. The calcium present in medium reacted with the fatty acids released by lipase to form insoluble white calcium salts. The lipase activity was determined as per standard method described by Prasad (2013), the absorption was measured at 405 nm. Enzyme units per ml (Uml^{-1}) of supernatant were calculated using the formula

$$Enzymatic\ activity = \frac{(A_s - A_b) \times V_f \times F_d}{0.018 \times T \times V_e}$$

where A_s: absorbance of sample at 405 nm; A_b: absorbance of blank at 405 nm; V_f: final volume of solution in ml; F_d: factor of dilution; T: total time taken during reaction; V_e: amount of supernatant in ml utilised in the reaction. At 405 nm, *p*-nitrophenol has an extinction coefficient of 0.018.

2.6. Estimation of richness using the distance-based operational taxonomic unit and richness (DOTUR)

The Shannon-Weiner diversity index was calculated as described by Magurran (1998). The species diversity in community were calculated using the formula

$$H = \sum [(p_i)] \times \ln (p_i)$$

where p = reflects the fraction of a unique phylotype in relation to the total number of unique phylotypes.

Evenness E was calculated as below:

$$E = \frac{H}{\ln(S)}$$

Where the phylotype richness is denoted by the letter S.

3. Result and discussion

3.1. Physico-chemical characteristic of the PPS

Physico-chemical properties of the PPS are presented in Table 1. The PPS was found slightly alkali with pH above 7. However, the electrical conductivity (2312 μmhoscm^{-1}) was found to be higher than the permissible limit (CPCB, 2017). The use of NaOH during pulping makes the PPS alkaline and high salt content was reflected in the elevated value of EC. In the pulp-paper mills industries, sodium hydroxide (NaOH) and sodium sulfite (Na_2S) were used for digestion of wood during pulping process for paper making. Presence of sulfate in the PPS might be due to the use of sodium sulphite during pulping process. The heavy metals, in particular Fe and Cu, were present in high concentrations (above the permissible limits) in the PPS, probably as a result of heavy metals accumulated in plants used as by the industry as raw materials (Yadav and Chandra, 2018). The results suggested the treated wastewater containing the PPS would not be suitable for irrigation.

Table 1 Physico-chemical parameters of pulp and paper mill sludge discharged after secondary treatment (PPS).

Parameters	Sludge	Permissible limits (EPA, 2003)
pH	8.65 ± 0.37	6.5 to 8.5
Electrical Conductivity (EC) (µmhoscm^{-1})	2312 ± 92.90	950
Humidity %	80.30 ± 3.88	–
Phosphate	192.30 ± 8.60	–
Sulfate	1329.00 ± 9.79	250
lignin	45,000 ± 678	–
Total nitrogen	203.30 ± 0.47	143
Total organic matter	58.88 ± 3.15	–
Na^+	658.00 ± 27.42	200
K_+	35.68 ± 1.58	–
Cl^-	05.53 ± 0.11	1500
Total heavy metals		
Cd	0.29 ± 0.012	0.01
Cr	0.35 ± 0.010	0.01
Cu	2.10 ± 0.012	0.05
Fe	7.34 ± 0.015	2.00
Zn	0.88 ± 0.009	2.00
Mn	0.68 ± 0.003	0.20
Ni	0.26 ± 0.002	0.10

All the values are mean ± SD (n = 3); unit of all parameters is in mgL^{-1} except pH, color and electric conductivity. Central Pollution Control Board (CPCB).

3.2. Characterization of organic pollutants in the PPS

The PPS is a complicated semi-aqueous system; it is challenging to analyse the organic chemicals present in it. Nevertheless, the GC–MS analysis of the ethyl acetate and DCM PPS extracts in this study confirmed the presence of low-molecular organic compounds, i.e., fatty acids, phenolics, esters, alkane, aldehyde, cyclic alkane, fatty alcohols and heterocyclic aromatic compounds, most of which were non- biodegraded (Fig. 1a–b; Tables 2 and 3). The ethyl acetate PPS extracts revealed a variety of peaks with varying retention times (RT) (Fig. 1a). Major peaks were noted at 9.47, 15.77, 14.39, 17.90, 17.93, 17.99, 19.70 and 21.31 RT and their ionization fractions were similar to the as thiocyanic acid; 1-ethyl-2-methyl cyclopentane; 8-pentadecanone; *cis*-1,3-dichloropropene; propanoic acid; wisanidine; methyl cyclooctane, and di-chloroacetic acid, respectively (Fig. 1a and Table 2). Presence of pentane and octane in the PPS may be due to their presence in the raw plant materials, while the toxicity of PPS could be enhanced due to acetic acid chlorination. For example, Mackulak et al. (2013) also reported the toxicity of chloroacetic acid.

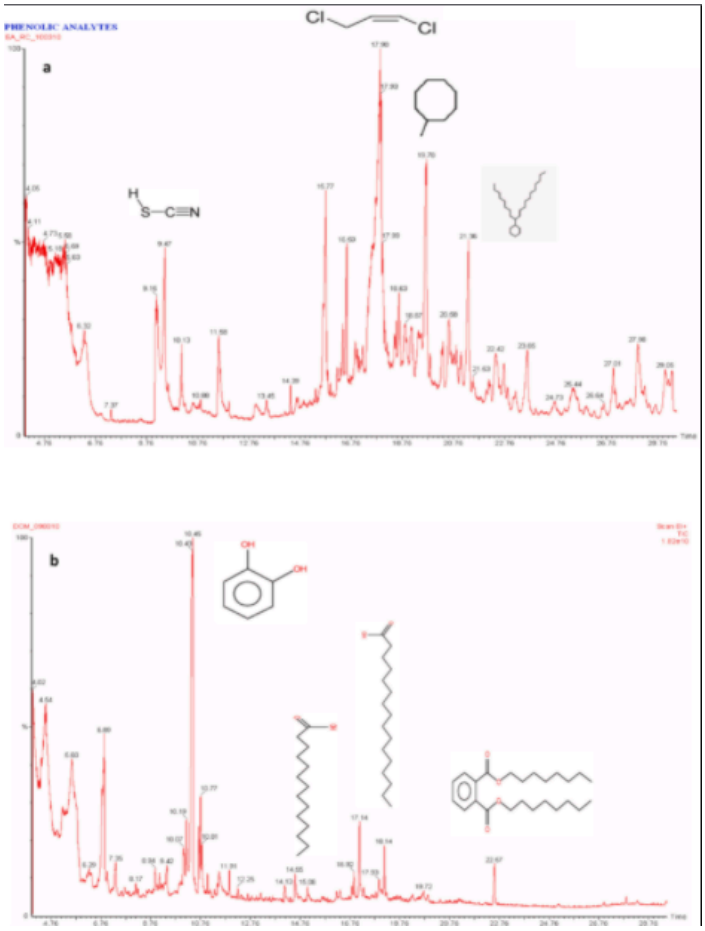

Fig. 1. Total ion chromatogram (TIC) of organic pollutants presents in ethyl acetate (a), and dichloromethane extract (b) of pulp and paper mill sludge after TMS

According to Chandra et al. (2011), bacteria cannot readily digest L-(+)-lactic acid and acetic acid. Besides, other peaks were also noted at RT 10.13, 11.58, 18.63, 18.87, 20.58, 22.42, 23.65, 27.01, 27.98 and 29.04 as guaiacol; D-*glycero*-D-manno-heptonic acid-4-lactone; 10-nonadecanone; 5,8,11-eicosatriynoic acid, methyl ester; 2,10- dimethyl-9-undecenal; 1,2-benzene di-carboxylic acid; (1,3-dimethylbutyl) cyclohexane; 1-octadecanol; 2,3-dihydroxypropyl ester of 9, 12,15-octadecadienoic acid (z,z) and 2-butyloxy carbonyloxy-1,1, 10-trimethyl-6,9-epidioxydecalin, respectively (Fig. 1a and Table 2). Guaiacol is a naturally occurring phenolic compounds derived from wood creosote preservatives and pyrolysis of lignin. Its presence concerning due to its potential carcinogenicity in humans. It has been declared by the European Chemical Agency (ECHA) as a substance of very high concern (National Center for Biotechnology Information, 2022).

Table 2 Compound identified by GC-MS analysis of PPS extracted with ethyl acetate.

S. No.	Retention time (RT) in min	Compounds
Fatty acid		
	7.37	Butyric Acid
	9.47	Thiocyanic Acid
	17.93	Propanoic acid
	18.87	5,8,11-Eicosatriynoic acid, methyl ester
	21.31	Dichloro acetic acid
Phenolics		
	10.13	Guaiacol
	16.43	2-methoxy-4-vinylphenol
	26.64	4-(1-E)-3-Hydroxyl-1-propenyl)-2-methoxy phenol
Ketones and Esters		
	11.58	D-*glycero*-D-manno-heptonic acid-4-lactone
	14.39	8-Pentadecanone
	18.63	10-nonadecanone
	27.98	9,12-octadecadienoic acid (z,z)-2,3-dihydroxypropyl ester
Polycyclic Aromatic Compounds		
	9.15	Purine, 9-(trimethylsilyl)
	19.09	Pyrene
Alkane and Cyclic alkane		
	15.77	1-ethyl-2-methyl-Cyclopentane
	24.73	3-ethyl-2-methyl-1-heptane
	17.90	*Cis*-1,3-Dichloropropene
	23.65	(1,3-dimethylbutyl) Cyclohexane,
	19.70	Methyl Cyclo-octane
	13.45	1-ethyl-trans-2-pentyl-cyclopropane
Aldehyde		
	20.58	2,10- dimethyl-9-undecenal
	21.53	Eicosane, 2-cyclohexyl
	22.42	1,2-Benzene dicarboxylic acid
Amides		
	10.26	Beta-ChloroEthylurea
	17.99	Wisanidine
	25.44	Acetamide, n-2-propynyl
Fatty alcohols		
	27.01	1-octadecanol
Miscellaneous		
	10.88	1,3,2,5-Dimethylene-*l*-rhamnitol
	22.73	Desulphosinigrin
	29.04	2-Butyloxy carbonyloxy-1,1,10-trimethyl-6,9-epidioxydecalin

Polycyclic Aromatic Compounds (PAC), i.e., purine, 9-(trimethylsilyl) (9.15) and pyrene (19.09) were also detected in the ethyl extracts. PAC is a broad family of organic molecules that are made up of two or more fused aromatic rings. These molecules need more attention because of their prevalence in the atmosphere and can impact negatively the ecosystems and human health (Tripathi et al., 2021 a). These are some of the most carcinogenic, mutagenic, and hazardous pollutants (Jaiswal et al., 2021). The Environmental Protection Agency and the European Union have designated several persistent organic pollutants as priority pollutants and their detection in the extracts confirmed that the secondary treatment was not sufficient in rendering the wastewater safe to be discharged or reuse. Therefore, further treatment is required prior to disposal.

Additional minor peaks were also observed at RT 7.37, 10.88, 13.45, 14.39, 21.53, 24.73 and 26.64 which were identified as butyric acid; 1,3,2,5-dimethylene-*l*-rhamnitol; 1-ethyl-trans-2-pentyl-cyclopropane; 8-pentadecanone; eicosane, 2-cyclohexyl; 3-ethyl-2-methyl-1-heptane and 4-(1-E)-3-hydroxyl-1-propenyl)-2-methoxy phenol, respectively. The

compound detected at RT 27.98 as monolinole in which is another name for 9,12-octadecadienoic acid (z,z)-2,3-dihydroxypropyl ester. These compounds are hepatotoxicants and potentially carcinogenic.

Interestingly, the compounds found in the DCM extracts showed major peaks at RT 10.43 and 10.45 as 4-*t*-octylphenol diethoxylate and catechol, respectively (Fig. 2b and Table 3). This indicated the importance of the choice of extractants. In order to build up a thorough picture of the organic pollutants present in complex matrices such as the PPS, a range of extractants should be used. The importance of Catechol is formed due to methylation of guiacol. Further some other small peaks were also observed at RT 8.17, 9.42, 10.07, 10.77, 10.81, 11.91, 12.25, and 14.13.14.55, 16.92, 17.14, 17.93, 18.14, 19.72 and 22.57 from DCM extract as propanoic acid; 2-hidroxy,methyl ester (methyl lactate); 1,3 bis (1,1-dimethylethyl)-1,1,3,3-tetramethyldisiloxane; l-alanin, n- (1-oxopentyl)-, methyl ester; 2-methyl-2,4-dimethoxybutane; 2-(2-(2- methoxyethoxy) ethoxy-1,3-dioxalane; 2-[2-ethoxyethoxy]ethoxy-trimethylsilane; glycerol; bicycle [2.2.1]hept-2-ene, 2-methyl; tetradecanoic acid; cinnamic acid; pentadecanoic acid; palmitelaidic acid; Hexadecanoic acid; octadecanoic acid and 2-oxabicyclo [3.3.0]oct-7-en- 3-one, 7-(1-hydroxy pentyl), respectively (Fig. 2b and Table 3).

Tetradecanoic acid in DCM extract has also been detected in hydro distillation of unsaponifiable matters of *Carduus pycnocephalus* by Chandra et al. (2018a, b). These pollutants showed antimicrobial activities against *Bacillus subtilis*, *Staphylococcus aureus* and *Mycobacterium smegmatis* (Abubaka and Majinda, 2016; Chaturvedi et al., 2021). As a result, these chemicals may inhibit microbial degradation in the environment and instead survive as persistent pollutant. The main identified components of DCM extract were n-fatty acids (C12 to C18), has a high preference for long chain *n*-alkanoic acids with even-numbered C atoms. Hexadecanoic acid (palmitic acid) was found to be most common compounds, which is corroborated with the findings of Morrison et al. (2001). These authors observed that palmitic acid was the most abundant fatty acid in extract from the fibres of numerous flax cultivars, its presence probably attributed to the raw materials used in the pulp-paper industry. Octacosanol was also has been reported as the major fatty alcohol found in the flax fibres (Qiu et al., 2020), in Niger seeds and linseeds (Deme et al., 2021; Chaturvedi et al., 2020a,b). The distribution of the aldehyde series match with that of free alcohols, as is typical in the plant kingdom, implying that aldehydes are intermediates in the production of alcohols from fatty

acids. Source of chlorophenols is likely the biocide used for wood treatment as it is not a natural lignin (Govarthanan et al., 2013, 2018).

Table 3 Compound identified by GC-MS analysis of PPS (DCM extraction).

S No.	Retention time (RT) in min	Compounds
Fatty acid		
	8.17	Propanoic acid, 2-hidroxy, methyl ester (methyl lactate)
	10.19	–
	11.04	2-oxovaleric acid
	14.55	Tetradecanoic acid
	15.05	Beta-hydroxypyruvic acid
	16.84	Hexadecanoic acid
	16.92	Petadecanoic acid
	17.14	Cinnamic acid
	17.93	Palmitelaidic acid
	18.14	Hexadecanoic acid
	19.72	Octadecanoic acid
Phenolics		
	10.43	4-*t*-octylphenol diethoxylate
	16.36	2-methoxy-4-vinyl phenol
	10.45	Catecol/citral
Alkane		
	10.77	2-methyl-2,4-dimethoxybutane
	10.81	2-[2-[2-methoxyethoxy] ethoxy-1,3-dioxalane
	11.53	2-propaneamine, 3-fluro-*N*-Phenyl
	11.91	2-[2-ethoxyethoxy]ethoxy-trimethylsilane
	14.13	Bicyclo [2.2.1]hept-2-ene, 2-methyl
Alcohol		
	12.25	Glycerol
Miscellaneous		
	9.42	1,3 bis (1,1-dimethylethyl)-1,1,3,3-tetramethydisiloxane
	10.07	l-Alanin, n-(1-oxopentyl)-, methyl ester
	22.57	2-oxbicyclo (3.3.0)oct-7-en-3-one, 7-(1-hydroxy pentyl)

3.3. Bacterial diversity in PPS

Six aerobic bacteria ITRRCPS1, ITRRCPS2, ITRRCPS3, ITRRCPS4, ITRRCPS5 and ITRRCPS6 were isolated from PPS. These bacterial strains were morphologically and biochemically identified (result not shown). The 16 S rRNA gene sequence similarity and blast analysis identified strains ITRRCPS1, ITRRCPS2, ITRRCPS3, ITRRCPS4, ITRRCPS5 and ITRRCPS6 as *Brevundimonas diminuta, Aeromonas punctata, Enterobacter hormaechei, Citrobacter braakii, Bacillus pumilus* and *Brevundimona sterrae* (Fig. 2), with the accession number HM172498, HM172499, HM172500, HM172501, HM172502 and HM172503, respectively. MEGA version 6 was used for phylogenetic and molecular evolutionary analysis (Tamura et al., 2013; Tripathi et al., 2021 c,d) and a phylogenetic tree was constructed for alignment of sequences. Figs. 2 and 3 are evolutionary connections of taxa the neighbor-joining approach was used to determine the evolutionary history of taxa. The proportion of duplicate trees in which the connected taxa grouped together in the bootstrap test (1000 repetitions) is indicated next to the branches. These culturable bacteria belonged

predominantly to the Proteobacteria phylum and are notable for their multidrug resistance (Benghait and Blaghen, 2020; Muraleedharan et al., 2019; Gou et al., 2020; Dong et al., 2020; Sharma et al., 2021 a & b and Scotta et al., 2011). However, there is little information on their ability to resist POPs. Results in this study indicate that these bacteria might also have developed resistant mechanisms against POPs and would warrant further in-depth investigation.

The restriction endonucleases *Taq*I and *Sau*3AI (Bangalore Genei, India) were employed to digest 16 S rRNA gene fragments obtained from bacterial populations living in pulp-paper mill effluent polluted sites in the RFLP analysis. Results showed the presence of six culturable bacterial species and seven non-culturable bacterial species (Aravinthan et al., 2015). Separate and combined 16 S rRNA fragments permitted the differentiation of total bacterial populations developing at polluted locations into Firmicutes and Proteobacteria, with each group showing a distinct 16 S rRNA RFLP pattern. Firmicutes, α-Proteobacteria, and γ-Proteobacteria were among the culturable bacterial populations in the PPS. The non-culturable bacterial communities also come under Firmicutes and two Proteobacteria clade-β and γ. The PPS samples showed γ-proteobacteria mainly *Klebsiella, Citrobacter, Bacteroides* were the abundant groups followed by β-proteobacteria and Firmicutes (Fig. 3). Firmicutes genera content mainly *Clostridia* in non-culturable condition while *Bacillus* was found most dominant strain in culturable condition (Govarthanan et al., 2013, 2018; Lee et al., 2013). Interestingly, it was found that β-proteobacteria were absent in culturable condition while α-proteobacteria were absent in non-culturable condition. *Citrobacter* was only genera which were found in both culturable and non-culturable condition (Figs. 2 and 3). *Citrobacter* from the γ-proteobacteria group were found in close proximity to Firmicutes in both cases. Low bootstrap values represented the significant diversity among the Firmicutes genera *Bacillus* and *Clostridia*. The abundance of *Aeromonas*, *Citrobacter*, *and Enterobacter* may be indicative of organic matter content and eutrophication (Tripathi et al., 2021e; Tripathi et al., 2022a, b). Results in this study showed the dominant composition of the non-culturable fraction of sludge microbiota which were not reflected in the culturable population, suggesting for a thorough understanding of the microbial diversity in the environment, there is a need of both using both culturable and non-cultural techniques. The findings also suggest that bacterial

populations may adapt differently under significant selection pressure due to the presence of a variety of persistent organic contaminants.

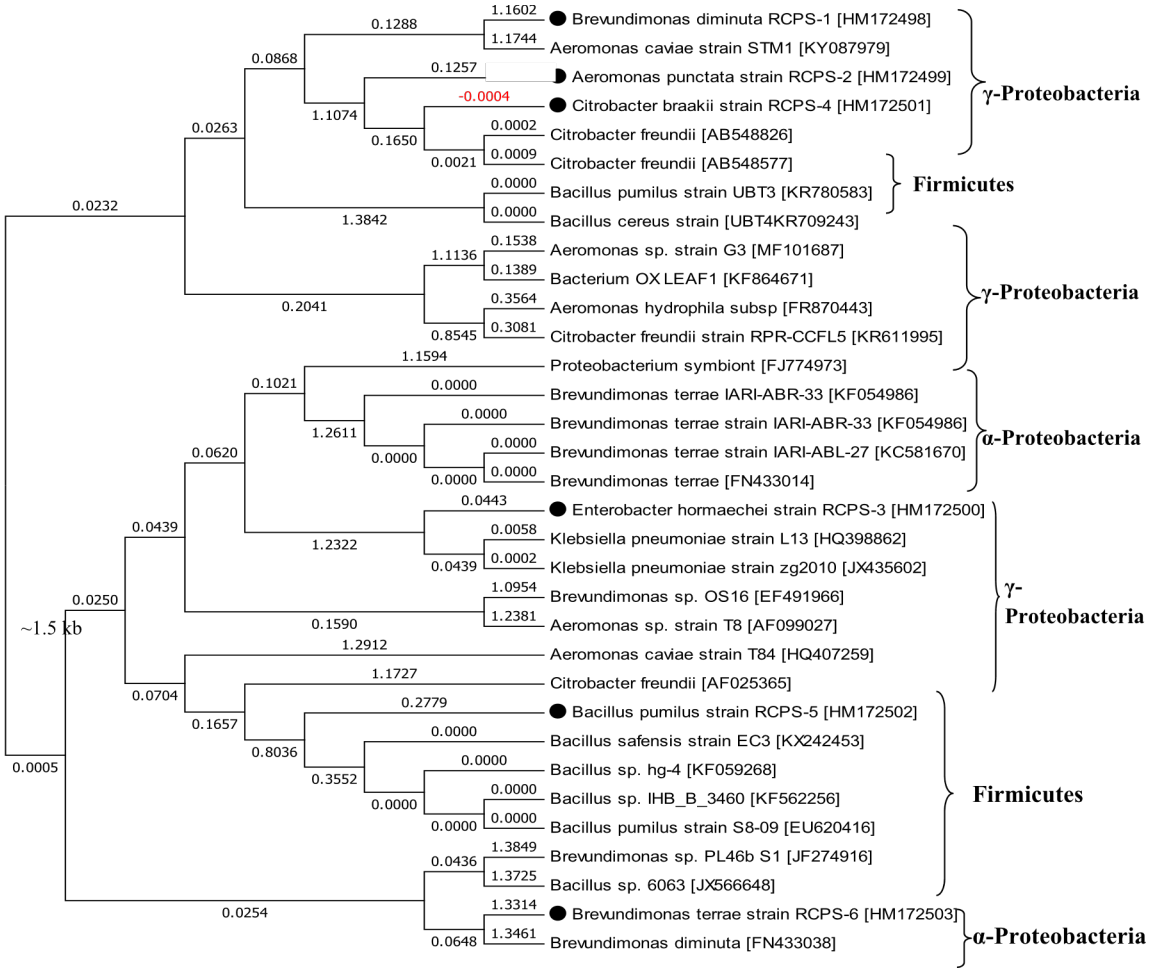

Fig. 2. The evolutionary connections of culturable bacterial species were inferred using the Neighbor-Joining approach. The ideal tree is presented, with a total branch length of 24.69218519. (near to the trees) The evolutionary distances were calculated using the Maximum Composite Likelihood technique and are in base substitutions per site. The study included 33 nucleotide sequences. The following codon locations were included: 1st+2nd+3rd + Noncoding. All spots with gaps and incomplete data were removed. The total number of places in the final dataset was 1414. MEGA6 was used to perform evolutionary analysis.

3.4. Lipase activity and quantification

All the bacterial strains were showed lipase positive activity i.e., they have capacity to degraded lipids. Further, activities were quantified with *p*-nitrophenol palmitate as a

substrate. *Bacillus pumilus* (RCPS-5; 6.34 U ml^{-1}) showed the highest lipase activity followed by *Aeromonas punctata* (RCPS-2; 6.34 U ml^{-1}) *>Citrobacter braakii* (RCPS-4; 5.55 U ml^{-1}) *>Enterobacter hormaechei* (RCSP-3; 5.32 U ml^{-1}) > *Brevundimonas diminuta* (RCPS-1, 4.85 U ml^{-1}) *>Brevundimonas terrae* (RCPS-6, 3.93 U ml^{-1}). The ability of *Bacillus cereus*103 PB to produce lipase and degrade palm oil mill effluent has been well-documented (Bala et al., 2014). Bacterial species Bacillus and Enterobacter showed Laccase and peroxidase which are helpful for reduction of pollutants (Yadav et al., 2011; Wang et al., 2015). *Bacillus pumilus* has been shown to produce proteolysis-resistant lipase (Sangeetha et al., 2014). The ability of the isolated strain to produce lipase would explain the presence of many of the fatty acids detected in the ethyl acetate and DCM extracts (Tables 2 and 3) where the plant lipids from the raw materials have been transformed to fatty acids.

3.5. Diversity index analysis

The diversity index is a useful tool for quantifying and describing the numerical organization of a community. Table 4 compares the diversity indices of the bacterial community using culture dependent and independent approaches. Gene functional patterns were shown to be related to wastewater temperature, dissolved oxygen and nitrogen levels, and organic loads. In this study, the number of culturable isolates was 8 and the non-culturable strains were 40. The PPS's culturable bacterial diversity has a low Shannon's diversity index (H) of 2.01. Non-culturable microorganisms had the highest value of 3.01 for PPS. While, the culturable and non-culturable isolates had species evenness (E) of 0.94 and 0.90, respectively. In pulp-paper mills effluent polluted sludge, the high values of diversity indices and evenness indicated a significant microbial diversity. Non-culturable bacterial communities detected in PPS had more species richness and diversity than culturable communities, but culturable communities had high evenness. Results in this study therefore showed that the highly polluted PPS had impacted on the bacterial community. The viable but nonculturable state enabled many bacteria in the environment to survive in adverse conditions such as the polluted PPS·As they are not viable and therefore not being detected by conventional methods, this explains the lower diversity index score from the culturable isolates. The higher species evenness scores also an indicated specific group of bacteria mainly from the Proteobacteria phylum, have developed mechanisms to survive in a high stress environment. As indicated above, the ability

of the isolated bacteria to resist POPs has not been widely explored and would require further studies.

Table 4 Comparison of richness, diversity and evenness of bacterial strains isolated from PPS.

S.No	Index	Culturable isolates	Unculturable bacterial strains
1.	S	8	40
2.	H	2.01	3.01
3.	E	0.94	0.90

Richness (S) = Total species in a community; Diversity $H = \sum[(p_i)] \times \ln(p_i)$, where p is the proportion of a distinct phylotype relative to the sum of all distinct phylotype; Evenness $E = \frac{H}{\ln(S)}$

4. Conclusion

This study demonstrated that persistent organic pollutants reach the environment via the disposal of pulp-paper mills sludge. Many the pollutants in the PPS were POPs and could create adverse effect in the biota. Results showed that the physico-chemical parameters were all above the permissible level, therefore, further treatment or improve treatment strategy is needed to minimize the environmental effect of pulp-paper mills waste as the secondary

culturable isolates were able to produce lipase that accounted for some of the fatty acids (e.g., palmitelaidic acid) detected in the PPS. Whilst the non-culturable bacterial communities had more species richness and diversity than culturable communities, but culturable communities had high evenness reflecting the ability of a few isolated strains to survive in the presence of heavy metals and POPs, whereas the non-culturable bacteria were able to withstand the harsh environmental conditions by entering a viable but non-culturable state. The isolates of this study have the potential to be utilised in the bioremediation of pulp-paper mills effluent but further work is needed to explore the ability of the isolates to tolerant POPs.

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Author statement

Sonam Tripathi; Conceptualization, Data curation, Investigation, writing; **Diane Purchase; Kaman Singh** and **Hind A. AL-Shwaiman**; review and revision; **Sangeeta Yadav** & **Ram Chandra**; Supervision, Funding acquisition, Conceptualization, Data curation, Investigation and Writing – original draft.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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