Influence of Long Term Irrigation with Pulp and Paper Mill Effluent on the Bacterial Community Structure and Catabolic Function in Soil

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Abstract Microbial communities play a vital role in maintaining soil health. A multiphasic approach to assess the effect of pulp and paper mill effluent on both the structure and function of microbial soil communities is taken. Bacterial communities from agricultural soils irrigated with pulp and paper mill effluent were compared to communities from soils irrigated with well water. Samples were taken from fields in the state of Uttarakhand, India, where pulp and paper mill effluent has been used for irrigation for over 25 years. Comparisons of bacterial community structure were conducted using sequencing of the 16S rRNA gene from both isolates and clone libraries attained from the soil. Community-level physiological profiling was used to characterize the functional diversity and catabolic profile of the bacterial communities. The multiphasic approach using both physiological and molecular techniques proved to be a powerful tool in evaluating the soil bacterial community population and population differences therein. A significant and consistent

difference in the population structure and function was found for the bacterial communities from soil irrigated with effluent in comparison to fields irrigated with well water. The diversity index parameters indicated that the microbial community in pulp and paper mill effluent irrigated fields were more diverse in both structure and function. This suggests that the pulp and paper mill effluent is not having a negative effect on the soil microbial community, but in fact may have a positive influence. In terms of soil health, this finding supports the continued use of pulp and paper mill effluent for irrigation. This is however only one aspect of soil health which was evaluated. Further studies on soil resistance and robustness could be undertaken to holistically evaluate soil health in this situation.

Keywords Catabolic profile · CLPP · Microbial community · Pulp and paper mill · 16S rRNA gene

Introduction

Soil health is dependent on a number of different functions, the main 4 being carbon transformations, nutrient cycles, soil structure maintenance, and the regulation of pests and diseases [1]. Soil microbial communities play a vital role in nutrient cycling, the decomposition of organic matter, carbon sequestration, soil genesis, soil structure and consequently water retention of the soil [2]. Therefore the study of soil community structure and function is an important aspect in assessing soil health. The microbial structural diversity of soil helps to describe a soil's capacity to recover from perturbations [3], where functional assessments give insight into the carbon and nutrient cycling capacities.

India has 406 registered pulp and paper mills [4], which are especially large consumers of fresh water (100–250 m³/

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ton paper). With practically all of this water reappearing as effluent (72–225 m³/ton paper), a large amount of treatment and disposal is required [5]. Pulp and paper mill effluent irrigation has been shown to increase crop yields through increasing nutrient status of the soil [6]. However, pulp and paper mill effluents also contain toxic elements such as magnesium, sodium, chlorides and sulphur as well as organic compounds such as chlorinated lignins and phenolic derivatives, which can cause toxic effects and nutrient imbalance in plants.

Several studies have shown pulp and paper mill effluent irrigation to increase the organic matter content in soil, thereby increasing the overall population of enumerable soil microorganisms [7, 8]. However, previous studies were based on culture-dependent methods (plate counting) which do not provide an accurate or full account of a soil bacterial community. The introduction of culture-independent techniques like 16S rRNA gene cloning directly from the environment have provided a more thorough insight into changes occurring in the structure of the microbial community as a whole [9]. Using these techniques a true account of community structure can be found.

Community-level physiological profiling (CLPP) is a technique which offers an easily applied protocol yielding information regarding mixed microbial community function, and functional adaptations over space and time. In CLPP heterotrophic bacterial communities are compared and classified based on sole carbon source utilization patterns (CSUPs) gathered using BIOLOGTM microplates [10]. CLPP has been widely and successfully used to obtain valuable information on microbial diversity in a number of different ecosystems and has been labeled as a useful tool in assessing soil health [1]. A combined multiphasic approach looking at both the function and structure of a soil community is now the preferred method when making a holistic assessment of soil health in the face of perturbations.

The aim of this study was to assess the effect pulp and paper mill effluent has on the structural and functional aspects on the microbial communities in soil used for agriculture. Bacterial community characterization was completed using CLPP, and 16S rRNA gene amplicon sequencing of both isolated culturable organism DNA and DNA directly extracted from soil microorganisms.

Materials and Methods

Field Sites

The effluent from Century Pulp and Paper at Lal Kuan, Uttarakhand, India (79°10'E longitude and 29°3'N latitude) undergoes primary and secondary treatment involving activated sludge process and discharge into a local

waterway canal containing fresh water. This effluent has been (last 25 years), and is currently being used as source of irrigation for sugarcane fields. Samples were collected from both the field irrigated with the pulp and paper effluent (designated as effluent irrigated field, EIF), and also from a neighbouring field irrigated with well water (designated as well-water irrigated field (WIF).

Soil Sample Collection and Characterization

Six composite samples, each made up of 10 sub-samples, were collected from both the fields (EIF and WIF) using a soil auger along zigzag paths (Zigzag sampling) to achieve random composite samples. The samples were collected at 0–20 cm depth during March 2010. The samples were transported to the laboratory in an insulated container at 4 °C and processed immediately.

The soil samples were air-dried at room temperature and sieved to 2 mm before analysis. Soil pH and electric conductivity (EC) were measured in a soil: water slurry (1:2, V/V) using digital pH and conductivity meters. The method described by Chopra and Kanwar [11] was employed for the analysis of organic C. Total N, available P and exchangeable K in soil samples were determined by elemental analysis according to Jackson [12]. Sodium accumulation in the soil was analysed as water-soluble sodium according to Rhoades [13].

Isolation and Identification of Culturable Organisms

Triplicate samples (1 g) were taken from each core sub-sample, homogenized in 10 ml of 0.85 % (wt/vol) saline, and serially diluted (tenfold) in the same. Aliquots (100 µl) were spread on high-nutrient-concentration tryptic soy broth agar (TSBA; Difco) and lower nutrient R2A medium for heterotrophs (Difco). From each sample colonies were picked at random from the plates and placed onto fresh plates. Once the purity of each of these cultures had been verified, the resulting isolates were stored as glycerol stocks at –80 °C.

Identification of Bacterial Isolates

Genomic DNA extraction from all the isolates was carried out as described by Pospiech and Neumann [14]. The gene encoding 16S rRNA from selected isolates was amplified by PCR using universal primer pair pA (5'-AGA GTT TGA TCC TGG CTC AG-3') and pH (5'-AAG GAG GTG ATC CAG CCG CA-3') [15]. Approximately 1 µg of PCR-amplified 16S rDNA gene fragments were cleaved with endonucleases *Dde*I, and *Taq*I (Fermentas, USA) separately at 37 °C for overnight and resolved by electrophoresis in 2.5 % agarose. Strong and clear bands were scored for similarity and clustering analysis using the software,

NTSYS-PC2 package (Numerical taxonomy analysis program package, Exeter software, USA).

Purified 16S rDNA of representative isolates from each cluster was used as a template in cycle sequencing reactions with fluorescent dye-labeled terminators (Big Dye, Applied Biosystems) using both primers pA and pH. All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases through a BLASTn search. Identification to the species level was determined based on the 16S rRNA gene sequence similarity ($\geq 97\%$) with that of a prototype strain sequence. Multiple sequence alignment of approximately 1,500-bp sequences was performed using CLUSTALW, version 1.8 [16]. A phylogenetic tree was constructed using the neighbor-joining method [17]. Tree topologies were evaluated through bootstrap analysis of 1,000 data sets using the MEGA 4.0 package [18]. 16S rRNA gene sequences were submitted to the NCBI GenBank database under accession numbers HM480304 to HM480359.

Extraction of Community DNA from Soil and Clone Library Construction

The microbial community DNA was also indiscriminately extracted (0.25 g of each sample in duplicate) using a power soil DNA kit (MO BIO, USA). Extracted genomic DNA was PCR amplified using the same pA and pH primer set and conditions as for the isolates. PCR products were ligated into the plasmid vector pCRII-TOPO (TOPO TA[®] Cloning kit Invitrogen) with subsequent transformation into electrocompetent *Escherichia coli* TOP10 cells (Invitrogen). All positive 16S rRNA gene inserts were grouped according to the patterns obtained after *Dde*I, and *Taq*I digestion. Unique RFLP patterns were identified visually and representatives of each restriction pattern were selected for 16S rRNA gene sequencing.

The MOTHUR platform [19] was used for removing chimeras and grouping the sequences as OTUs. The 16S rRNA gene sequences having similarity $\geq 97\%$ were considered as the same OTU (or phylotype or ribogroup). From the combined grouping, only one representative clone from each OTU was taken for phylogenetic analyses. Representative sequences have been assigned GenBank accession numbers HQ166629 to HQ166707.

Community Level Physiological Profiles (CLPP)

The samples were suspended in 10 ml of phosphate buffer and shaken for 3 h at room temperature. Aliquots (2 ml) of the resulting suspension were diluted in buffer to adjust the optical density (420 nm) to be close to 0.2 which were then used as the inoculum for the microplates. Each well of the BIOLOG EcoPlate[™] (Biolog Inc., Hayward, CA, USA)

was inoculated with 150 μ l of inoculum and incubated at 25 °C. Absorbance readings were taken periodically (every 6 h for a total time period of 168 h for both WIF and EIF soil samples) at 590 nm with a Microlog plate reader.

Analysis of CLPP data was completed according to Weber et al. [20] and Weber and Legge [21]. Analysis of the well colour development over time showed fairly consistent development for all field plots, therefore absorbance at 72 h was chosen as the metric for analysis. Principle component analysis (PCA) was completed using Statistica 8.1 using the covariance matrix of the CSUPs from all of the data gathered. Plate triplicates were averaged before all analyses.

The Shannon index which is a measure of what is often called “diversity” is a common ecological metric used to track and understand shifts in communities over space and time. Using the CSUP gathered from a single BIOLOG[™] plate, substrate diversity (H) can be calculated as:

$$H_{CLPP} = - \sum p_i \ln(p_i)$$

where H_{CLPP} substrate diversity, p_i ratio of the activity of a particular substrate to the sums of activities of all substrates activity—chosen metric for analysis (absorbance value (590 nm) at 72 h)

Another parameter substrate richness (S), which is a measure of the number of different substrates utilized by a microbial population and is calculated as the number of wells with a corrected absorbance greater than 0.25.

Results and Discussion

Irrigating crops with pulp and paper mill effluent is a cheap and attractive alternative to the discharge of this effluent into natural waterways. Although several previous studies have investigated the effects of pulp and paper mill effluents on the structure of soil microbial communities, these studies were based solely on biochemical and culture based methods [7, 8]. The multiphasic approach combining CLPP, culturable population, and 16S rRNA gene cloning analyses used herein showed an encompassing account and representation of the bacterial response to the pulp and paper irrigation than any single approach could have alone.

Effluent irrigated field soils had higher pH, EC and organic C compared to WIF soils (Table 1). Total N, available P and exchangeable K of EIF soils were respectively 92, 167, and 46 % higher than WIF soils. The exchangeable Na content of the soils irrigated with the treated pulp and paper effluent was 251 % higher than for the WIF soils. These observations are similar to those observed by Kannan and Oblisami [7] and Tripathi et al. [8]. Given the neighbouring location of these 2 fields it is reasonable to believe that the consistent (25 years) of irrigation

Table 1 Characteristics of WIF and EIF soils

Soil characteristics	WIF ^a	EIF ^b
Soil type	Sandy loam	Sandy loam
pH	7.25 ± 0.11	8.43 ± 0.12
EC (dS m ⁻¹)	0.24 ± 0.07	0.81 ± 0.11
Organic carbon (%)	0.31 ± 0.09	2.14 ± 0.22
Total N (mg l ⁻¹)	118.67 ± 13.48	228.72 ± 18.34
Available P (mg l ⁻¹)	13.21 ± 3.66	35.28 ± 4.77
Exchangeable Na (mg l ⁻¹)	68.82 ± 5.62	242.12 ± 13.45
Exchangeable K (mg l ⁻¹)	171.23 ± 10.03	251.13 ± 14.47

^a Water irrigated fields^b Effluent irrigated field

Fig. 1 Phylogenetic tree based on the 16S rRNA gene sequences of bacterial isolates from EIF and WIF and their closest relatives, created by the neighbour-joining method. The numbers on the tree indicate the percentage of bootstrap sampling derived from 1,000 replicates. 16S rRNA gene sequence of *Ferroplasma thermophilum* used as an out-group

has been the driver for this observed difference in soil characteristics. The higher levels of nutrients found in the effluent irrigated soils can be seen as a positive aspect of this effluent irrigation methodology. A multiphasic assessment of the microbial community was also completed to gain a better understanding of the effect these documented nutrient changes, and other stressor effects from the pulp and paper mill effluent, had on the health of the soil.

Phylogenetic Analysis of Bacterial Isolates

A total of 128 isolates were selected using a random routine (71 from WIF and 57 from EIF) for further analysis.

Cluster analysis of 16S rRNA gene restriction patterns based on the Jaccard's similarity index grouped these isolates into 20 distinct groups. It can be seen that both the

EIF and the WIF fields were dominated by *Bacillus* and *Bacillus*-derived genera. These genera have been reported inhabiting industrial effluents [22], and chemically

Fig. 2 Neighbour-joining phylogenetic tree of 16S rRNA gene sequences of clones of *Proteobacteria* from EIF and WIF fields. 16S rRNA gene sequence of *Ferropasma thermophilum* used as an out-group

contaminated water samples [23]. They play a potential role in kraft lignin degradation [24], and therefore it is not with great surprise that these genera were found in the effluent irrigated samples. It should however be noted that the identification of *Bacillus* in the water irrigated fields suggests that they are ubiquitous to the soil in this region and that the pulp and paper mill in fact had nothing to do with the proliferation of these bacterial groups.

The other genera identified were *Amycolaptosis*, *Staphylococcus*, *Flavobacterium*, *Streptomyces*, *Arthrobacter*, *Rheinheimera*, and *Pseudomonas*. A phylogenetic reconstruction of these isolates sequences, together with sequences from their closest relatives is shown in Fig. 1. No major groupings could be based on EIF or WIF samples alone, the EIF and WIF samples are intermixed suggesting that based on this data no large shift in population has

Fig. 3 Neighbour-joining phylogenetic tree of 16S rRNA gene sequences of clones of remaining phyla from EIF and well WIF fields. 16S rRNA gene sequences of *Ferroplasma thermophilum* used as an out-group

occurred in response to the pulp and paper mill effluent irrigation.

Phylogenetic Analysis of 16S rRNA Gene Clone Library

From the bacterial 16S rDNA clone library, 258 clones (115 from WIF and 143 from EIF) were randomly selected and each clone was subjected to PCR–RFLP. A total of 82 (37 from WIF and 45 from EIF) different restriction patterns were detected and sequenced. A total of 71 OTUs were detected based on sequences having similarity of $\geq 97\%$. The sequenced clones were affiliated with sequences from 10 phyla of which one (OP10) is unculturable. Other phyla were Alpha, Beta, Gamma-, and Delta- subdivisions of *Proteobacteria*, *Acidobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Cyanobacteria*, *Chloroflexi*, *Gemmatimonadates*, and *Aquificae*. A phylogenetic reconstruction of these clone sequences, together with sequences from their closest relatives is shown in Figs. 2 and 3.

A large proportion of the clones in EIF library (47 %) were identified as *Betaproteobacteria*, related to the genera *Thiobacillus*, *Leptothrix*, *Aquabacterium*, *Aquicola*, *Curvibacter*, *Azohydromonas*, *Azoarcus*, *Ideonella* and *Denitratisoma*. Fierer et al. [25] observed that the *Betaproteobacteria* exhibited copiotrophic attributes; their relative abundances were highest in soils with high C availability. It was also reported that in aquatic environments, *Betaproteobacteria* are commonly associated with substrates rich in organic carbon [26]. These previous works collectively corroborate our finding with regards to the dominance of *Betaproteobacteria* in the EIF. A possible explanation for this dominance can be explained due to the wider range of organic substrates and increased nutrient status of the pulp and paper mill irrigated soil.

Comparatively, WIF clone library was dominated by *Acidobacteria* (36 %). It is well known that the *Acidobacteria* phylum are most abundant in soils with very low resource availability and their relative abundances are lowered in an individual soil amended with high concentrations of organic C [25].

The Shannon-Wiener diversity Index (H) showed that the microbial community in EIF samples were more diverse compared to those from the WIF, whereas evenness (E) was quite consistent in both fields (Table 2). This again supports the idea that the bacterial communities in the EIFs have adapted to and are thriving due to the higher nutrient and carbon loads found in the pulp and paper effluent. This is a positive result as diversity has been linked to soil health and robustness [3], and also it seems that bacterial communities present in EIFs managing the paper mill effluent pollutants as additional carbon source [27]. It seems that the pulp and paper mill effluent has not adversely affected

Table 2 Diversity indices of isolate and clone libraries based on 16S rDNA sequences

Type of data	Clone library data		Isolate library data	
	EIF	WIF	EIF	WIF
Shannon-Wiener diversity index	3.721	3.476	2.814	2.518
Evenness	0.995	0.994	0.955	0.981

the soil community and in fact the structural diversity has increased. This finding is encouraging however is evaluating only one aspect of soil health.

Community Level Physiological Profiling

In CLPP analysis, a clear difference between the two different types of field plots can also be seen via PCA ordination of the CSUPs (Fig. 4), lending further support to the idea that bacterial community has adapted of the to the pulp and paper effluent. This was also evident from an increase in AWCD, richness, and diversity (Table 3). Guild analysis showed that in comparison to the WIF, the EIF bacterial communities proportionally utilize carbohydrates slightly more heavily, and amines/amides slightly less heavily (Fig. 5). Both results can be explained as an adoption of the bacteria to a carbohydrate rich pulp and paper effluent.

The soil community has shifted both its structure and function based on the pulp and paper mill effluent. The shift however was in the positive direction showing greater functional and structural diversity. Based on these results it is suggested that pulp and paper mill effluent does not adversely affect the soil microbial community, and in fact may enhance its attributes.

Fig. 4 Principle component analysis (PCA) ordination of the CSUPs gathered using BIOLOGTM ECO plates. Prefix E and C represents samples from EIF and WIF respectively

Table 3 Diversity indices based on community level physiological profiling (CLPP) *p*-values based on 2-tailed paired unequal variance *t* tests

Sample	AWCD	Richness	Diversity
WIF	0.844 ± 0.108	21 ± 0.2191	3.042 ± 0.054
EIF	1.144 ± 0.103	26.278 ± 1.290	3.211 ± 0.034
<i>p</i> -value	0.0005	0.0009	0.0001

Fig. 5 Percent of total carbon source utilization response, for the different CSUP guilds- amines and amides, amino acids, carboxylic and acetic acids (C&AA), carbohydrates (carbs), and polymers. Prefix E and C represents samples from EIF and WIF respectively

Conclusions

It was found that the microbial communities in pulp and paper mill EIFs were more diverse in both structure and function when compared to those in water irrigated fields. The multiphasic approach integrating physiological, biochemical and molecular methods yielded a holistic picture of the effects of pulp and paper mill effluent on agricultural soil microbial communities. The results suggest that pulp and paper mill effluent does not have a negative influence on the microbial community in the agricultural soil studied, and therefore using pulp and paper mill effluent for irrigation is a promising water reuse method. Microbial community structural and functional diversity are important measures of soil health, however additional resistance and robustness measures could be further studied in order to gain a better idea of the full effect of pulp and paper mill effluent on overall soil health.

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