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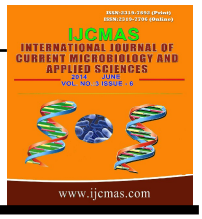

Original Research Article

Microbial community PLFA responses to ecosystem restoration in a chronosequence coal mine overburden spoil and implications of soil quality

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Keywords

PLFA, microbial community structure, mine overburden spoil, reclamation.

A B S T R A C T

PLFA profiling provides a set of molecular markers for different microbial taxa, and considered as a robust tool that consistently discriminates microbial communities of different origin and land management strategies involved in ecosystem structure and function. The relative distribution of 59 PLFAs elucidates significant differences in microbial community structure in different coal mine overburden spoil with variation in Shannon diversity index from 2.2629 (OB_6) to 2.8385 (OB_8). Higher Pielou's evenness index in NF (0.7197) and OB_{10} (0.7404) suggested greater diversity than OB_0 (0.7285). Ratio of gram-positive to gram-negative exhibited a decline trend from OB_0 (7.478) to OB_{10} (1.795). Fungal-to-bacterial ratio exhibited an increasing trend from OB_0 (0.0216) to OB_{10} (0.0990), and was highest in NF (0.1128). F:B ratio showed positive correlation with pH ($r=0.966$, $p<0.001$), moisture ($r=0.784$, $p<0.001$). Principal component analysis and cluster analysis can able to discriminate microbial community structure into independent clusters. Redundancy analysis can contribute for soil quality assessment based on changes in microbial community structure and relative distribution of 59 PLFAs. PLFA profiling provide an approach for microbial community assessment and their impacts on environmental processes, which represent *in situ* soil conditions and therefore can be used for monitoring restoration of coal mine spoil.

Introduction

Surface coal mining activities have left fertile native forest soil into a degraded, disequilibrated geomorphic system, which lead to the loss of vegetation, creation of barren pits and overburden of mine spoil. The successful restoration of coal mine overburden spoil through remediation should be not only to remove (or stabilize) the contaminants, but also to restore the capacity of mine spoil to function at its full potential, productive and a sustainable ecosystem. The concept of pedodiversity as a way to describe soil spatial diversity, including its link to biodiversity and landscape ecology has emerged as important determinants of ecosystem

processes. Therefore, the criteria for restoration of mine spoil largely focused on careful consideration of all the components of the plant-soil system (Mummey *et. al.,* 2002; Claassens *et al.,* 2006; Urbanova *et al.,* 2011), because the patterns observed aboveground are actually being driven by the belowground processes (He *et al.,* 2013).

Soil microbiological characterization (number, respiration rate and enzyme activities) provide an estimate of total pool size and gross activity measures, which can not be treated as sensitive indicators because of the redundancy of functions and complex interactions within the communities. However, soil microbial communities are the driving force mediating different soil processes such as organic matter decomposition, biogeochemical cycles, bioremediation and fertility (Zelles, 1999), nutrient cycling that regulate its size, activity and structure. Changes in microbial community structure are known to occur even though the total microbial community size remains unchanged (Renella *et al.,* 2008), which suggested that the microbial community structure may be used as a sensitive bioindicator of disturbance and reclamation progress as compared to the measures of either general microbial processes or overall community size (Veresoglou *et al.,* 2011).

Soil microbial community structure may be used to determine biodiversity, ecological processes and structure, ecosystem sustainability, and respond much faster to stress/disturbances (Kujur and Patel, 2014). Further, measurement of microbial community can be used to assess the status of microbial ecosystem, and in that sense the quality/potential of mine overburden spoil, and the progress of restoration.

However, the relative distribution of microbial communities and their activities regulating ecosystem function directly or indirectly was influenced by several factors. Soil microbial community composition varies in accordance with their physiological and nutritional status, environmental factors such as soil textural composition (Buyer *et al.,* 2002; Girvan *et al.,* 2003; Ulrich and Becker, 2006; Johnson *et al.,* 2006; Lauber *et al.,* 2008), aggregate size, temperature (Heipieper *et al.,* 1996; Pietikainen *et al.,* 2000), soil pH (Persson *et al.,* 1989; Baath *et al.,* 1995; Merila *et al.,* 2002; Baath and Anderson, 2003; Fierer and Jackson, 2006; Lauber *et al.,* 2008), moisture content (Sajbidor, 1997; Meimei *et al.,* 2008; Marais *et al.,* 2012; Moyano *et al.,* 2013; Zhou *et al.,* 2014), soil pH (Baath and Anderson, 2003; Rajapaksha *et al.,* 2004; Fierer and Jackson, 2006; Claassens *et al.,* 2011), organic C, total N and extractable P (Lauber *et al.,* 2008; Merila *et al.,* 2010), heavy metal toxicity and chemicals (Frostegard *et al.,* 1993; Heipieper *et al.,* 1996; Kandeler *et al.,* 2000: Rajapaksha *et al.,* 2004).

Besides, the microbial community structure is governed by the interactions between plant, climate and management practices (Bardgett and McAlister, 1999; Steer and Harris, 2000), plant inputs of litter and exudates, vegetation (Kowalchuk *et al.,* 2002; Potthoff *et al.,* 2006; Meimei *et al.,* 2008; Buyer *et al.,* 2010; Yu and Ehrenfeld, 2010; Ben-David *et al.,* 2011; Chao *et al.,* 2013), enzyme activity (Maharana and Patel, 2013b) in different soil profiles, which can shift the lipid composition (Denich *et al.,* 2003). Further, soils possessing higher microbial diversity is the characteristic feature of fertile ecosystem, whereas degraded soil are

characterized with low microbial diversity that often hardly responds to the environmental changes. Therefore, the relationship between microbial community structure and ecosystem function has attracted considerable research interest.

The occurrence of higher microbial diversity and difficulties in culturing native microorganisms make culture-based methods inadequate and inefficient for differentiating soil microbial communities. Therefore, a culture-independent approach is used to determine soil microbial community *i.e.* phospholipid fatty acids analysis (PLFA) of microbial membranes (Tunlid and White, 1992; Zelles *et al.,* 1992; Frostegard *et al.,* 1996, 2011), which become an important ecological tool not only to determine viable microbial biomass (Bardgett and McAlister, 1999), but also the shift in microbial community structure as well as the soil nutritional/physiological status (White *et al.,* 1997; Kujur and Patel, 2014). Phospholipid fatty acids (PLFAs) are potentially useful signature molecules exclusively found in cell membranes of microorganisms, and component phospholipid fatty acids are rapidly metabolized following cell death, not found in storage lipid/anthropogenic contaminants and have high turnover rate (Tunlid and White, 1992). Besides, PLFAs have several features that reinforce their use as indicator of environmental stress, which allows them to respond both intracellular and extracellular environment conditions, and hence can be used as indicator of environmental monitoring and assessment (Heipieper *et al.,* 1996).

Phospholipid consists of a single molecule of glycerol (3C alcohol), with two OH groups being replaced by two fatty acids by ester or ether linked (hydrophobic tail), and third OH group by a phosphate group (hydrophilic head). Microbial fatty acids are typically C_{12}-C_{24} long, but the membrane fatty acids are usually C_{14}-C_{20} long (Morgan and Winstanley, 1997). PLFA can be classified into ester-linked phospholipid (EL-PLFAs, 60-90%) and non-ester linked phospholipid (NEL-PLFAs, 10-40%) fatty acids. The ether-linked phospholipid fatty acid is rare, but has been found in *Archaea*. EL-PLFAs are further subdivided into ester-linked unsubstituted (EL-UNFAs) and hydroxyl substituted (EL-HYFAs) fatty acids. EL-UNFA includes saturated (EL-SATFA), monounsaturated (EL-MUFA) and polyunsaturated (EL-PUFA) fatty acids. EL-SATFA has two sub-groups: branched chain (BRANCs) and straight chain (STRAs) fatty acids. NEL-PLFAs include unsubstituted (NEL-UNFA) and hydroxyl substituted (NEL-HYFA) fatty acids (Zelles, 1999).

The PLFA profiles provide a broad diversity measurement of microbial community at the phenotypic level for rapid characterization of broad taxonomic groups of soil microbes, although it cannot be linked to microorganisms at species level (Zelles *et al.,* 1992; Frostegard *et al.,* 1993). The different subsets of the microbial community have various PLFA patterns with varying chain length, saturation and branching, which can be used as 'microbial community fingerprint' (Vestal and White, 1989; White *et al.,* 1996; Zelles, 1999; Steer and Harris, 2000). The phospholipids of microbial groups contain a variety of unique fatty acids, which can serve as biomarkers (Kaur *et al.,* 2005). The fatty acid extracted from sediments can able to classify distinct microbial groups: micro-eukaryotes (PUFA), aerobic prokaryotes (MUFA), gram-positive and anaerobic

bacteria (saturated and branched fatty acids; C_{14} to C_{16}), branched-chain fatty acids (*iso* and *anteiso*) are characteristic for gram-positive bacteria. Gram-negative bacteria contain unique hydroxyl fatty acids in the lipid portion of lipopolysaccharides in cell wall. LPS-OH fatty acids were used as an indicator of gram-negative bacteria in environmental samples (White, 1994). The total amount of PLFAs was used to indicate the total microbial biomass and the sum of PLFAs (14:0, 15:0, 16:0, 17:0, 18:0, 18:1ω9c, 20:0, 21:0, 22:0, 24:0) was considered to be predominantly of bacterial origin (Vestal and White, 1989; Tunlid and White, 1992). The PLFAs (16:1ω7c, cy17:0, 18:1ω7c cy19:0) are the representatives of heterogeneous groups of soil microorganisms most prevalent in gram-negative bacteria (Zelles *et al.,* 1997; Díaz-Ravina *et al.,* 2006; Lores *et al.,* 2010; Dicken *et al.,* 2013), the *iso* and *anteiso* branched PLFAs (a13:0, a14:0, i14:0, a15:0, i15:1ω6c, a15:1ω9c, a16:0, a17:0, a17:1ω7c, i17:1ω9c) typically represents gram-positive bacteria (Zelles, 1999; Lores *et al.,* 2010; Dicken *et al.,* 2013).

Besides, the sulfate-reducing bacteria including other anaerobic bacteria were represented by saturated and branched (C_{16} to C_{19}) fatty acids (Morgan and Winstanley, 1997). PLFAs (18:1ω9c, 18:2ω6c, 18:3ω6c) are used to represent common fungi (Frostegard and Baath, 1996; Myers *et al.,* 2001; Lores *et al.,* 2010; Dickens *et al.,* 2013), but linoleic acid (18:2 ω6) is a good indicator of fungi and fungal biomass (Frostegard and Baath, 1996). Besides, PLFA 18:2ω6 was also used as an indicator of higher eukaryotic organisms such as plants (Olsson *et al.,* 1999). The unsaturated fungal biomarker 16:1ω5c is typical for arbuscular mycorrhizal fungi (Olsson, 1999; Dickens *et al.,* 2013). The methyl branched PLFAs 10Me16:0, 10Me17:0, 10Me17:1ω7c, 10Me18:0, 10Me19:1ω7c, 10Me20:0 representing actinomycetes (Kroppenstedt, 1985; Zelles, 1999; Hill *et al.,* 2000), PLFAs 14:1ω7cDMA, i15:0DMA, 16:1ω7cDMA, 18:0DMA, 18:2DMA, 19:0cy for anaerobes (Frostegard *et al.,* 1991; Zelles, 1997; Zelles, 1999), PLFA 16:1ω7c, 18:1ω7c for aerobic bacteria (Zhong *et al.,* 2009), PLFA (16:1ω7c, 16:1ω8c) for methanobacter (Bowman *et al.,* 1991; Hill *et al.,* 2000), PLFA i17:1ω7c, 11:1ω6c, 10Me 16:0 (Robie and White, 1989) and PLFA 10Me18:0 (Zelles, 1997, 1999) for sulphate reducing bacteria.

The shift in PLFAs can reflect overall changes in microbial community structure, which can be used as an indicator of disturbances and provide valuable information regarding reclamation process (Arshad and Martin, 2002; Claassens *et al.,* 2006; Renella *et al.,* 2008; Chowdhury *et al.,* 2011; Veresoglou *et al.,* 2011). The chronosequence coal mine overburden spoil would be associated with characteristics microbial community structure. However, the nearby undisturbed forest soil supported by specific microbial community with defined environmental requirements was taken as reference, which could be used to facilitate interpretation of microbial community structure derived from the chronosequence coal mine overburden spoil in order to monitor ecosystem restoration. Besides, if this ecosystem was resilient, the microbial community structures in chronosequence coal mine overburden spoil would approach that of the nearby forest soil through succession. Keeping the above facts, the present investigation was designed to provide a

comparative assessment of microbial community structure in six different coal mine overburden spoil in chronosequence with respect to nearby forest soil, which can be used as a valid monitoring tool to access the efficacy of reclamation process. The fungal to bacterial PLFAs, and gram-positive to gram-negative PLFAs ratio within the microbial community were estimated in order to understand the relationship between microbial community structure and ecosystem function. Further, the soil characteristics important in driving succession by shifting microbial community structure during restoration of coal mine overburden spoil to nearby forest soil was determined.

Materials and Methods

Study site

The present study was carried out in the Basundhara (west) open cast colliery in the Ib valley of Mahanadi Coalfields Limited (MCL), Odisha, India (Geographical location: 22º 03' 58" - 20º 04' 11" north latitude and 83º 42' 46" - 83º 44' 45" east longitude). The coal mine overburden spoil have been grouped into six different age series (fresh: OB_0, 2 yr: OB_2, 4 yr: OB_4, 6 yr: OB_6, 8 yr: OB_8 and 10 yr: OB_{10}) since inception (Figure 1). Tropical dry deciduous forest was considered to be the natural vegetation of the site, which experiences a semi-arid climate (1300 mm rainfall y^{-1}, annual average temperature 26°C, and relative humidity 15%) with three distinct seasons *i.e.* summer, rainy and winter.

Soil sampling

Sampling was done in accordance with the general microbiological procedure (Parkinson *et al.*, 1971) three times representing three seasons during the study period *i.e.* summer (April), rainy (July) and winter (January). Each coal mine overburden was divided into 5 blocks, and from each block five spoil samples were collected randomly from (0-15) cm soil depth by digging pits (15 x 15 x 15 cm^3) referred to as 'sub-samples'. The sub-samples collected from each block of an overburden were thoroughly mixed to form one 'composite sample'. Thus from each overburden, five composite mine spoil samples were collected. Similar sampling strategies were followed for different coal mine overburden (OB_0, OB_2, OB_4, OB_6, OB_8 and OB_{10}) as well as the nearby native forest soil (NF). The composite samples were homogenized, sieved (0.2 mm) and stored at 4°C until analyzed.

Phospholipid fatty acid (PLFA) analysis

Lipids extraction was performed and subjected to fractionation and quantification using the procedure described by Buyer *et al.* (2010), which is based on that of Bligh and Dyer (1959) as modified by White *et al.* (1979). Lyophilized soil sample (5g) dry weight was sonicated with a mixture containing phosphate buffer, methanol and chloroform (4:10:5 v/v/v) for 10 mins in a sonicating water bath at room temperature, and was rotated end over end for 2hr at room temperature. The mixture was subjected to centrifugation at 2500 rpm for 10 mins, and the liquid phase was transferred followed by the addition of equal volumes of distilled water and chloroform (5:5 v/v), shaken vigorously, and incubated for 24hr for separation of two phases. The bottom (organic phase) was evaporated under nitrogen (N_2) and stored at -20^{0}C. Then the lipid classes were separated by solid phase extraction (SPE) chromatography by washing the

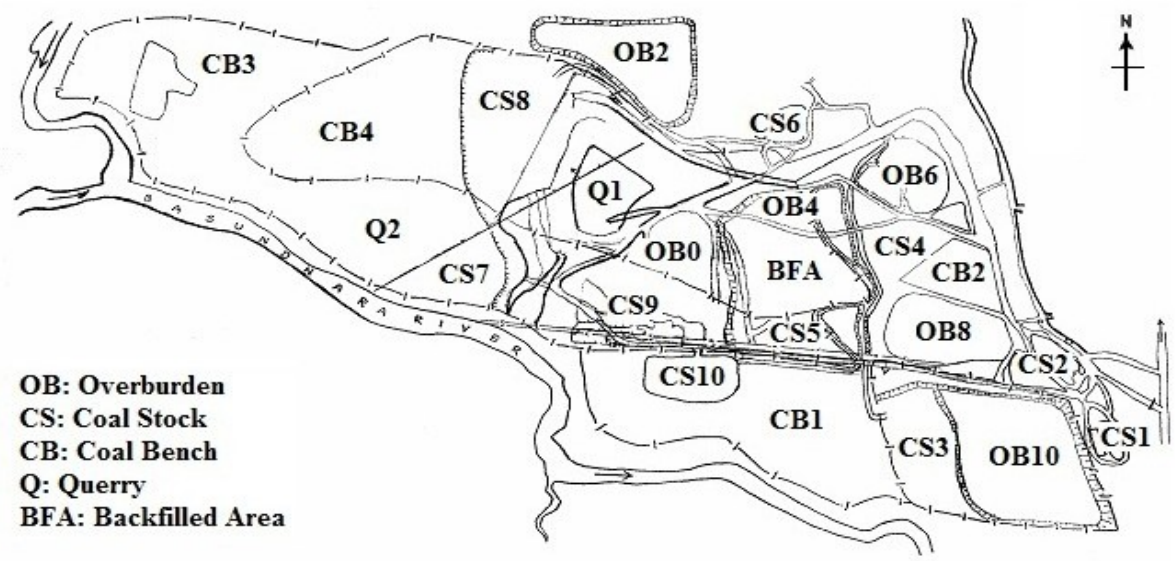

Figure.1 Site map of Basundhara (west) open cast colliery, Sundargarh

silica gel column with chloroform and then, after loading the extract in chloroform the neutral, glyco and phospholipids were eluted with chloroform, acetone and methanol respectively followed with the evaporation of the phospholipids fraction under N_2 and storing at -20^{0}C. The trans-esterification of fatty acids was performed with equal volumes of methanol and toluene (1:1 v/v) following a mild, alkaline methanolysis of phospholipids by methanolic KOH at 37^{0}C for 15mins. The resulting ester-linked fatty acid methyl esters (FAME) was dissolved in a mixture containing isooctane or hexane, acetic acid and double distilled water (2:0.3:2 v/v/v) and vortexed in order to separate the phases. The top (organic) phase was removed following the extraction process repeatedly with addition of hexane, and the combined phase was evaporated under N_2 and stored at -20^{0}C. The extracts were cleaned up by SPE using an NH_2 SPE column and the samples were dissolved in equal volumes of hexane: methyl tert- butyl ether (1:1 v/v) and was quantified by GC-MS. This method is simple, fast and is used for the analysis of soil microbial community including non-culturable microbes.

PLFA nomenclature follows the common convention of A:BωC (White *et al.,* 1997), where the total number of C atoms in the fatty acids is denoted as 'A', and the number of double bonds as 'B'. The position of the double bond is defined by a symbol ω followed by the number of carbons 'C' from the methyl end of the fatty acid molecule. The prefixes *cis* and *trans* configuration are indicated by c and t; i and a refer to *iso* and *anteiso* branching; *br* indicates an unknown methyl branch position; *cy* refers to cyclopropyl fatty acids. Hydroxy groups are indicated by 'OH'. 10Me indicates a methyl group on the C_{10} atom from the carboxyl end of the molecule (Baath and Anderson, 2003; Steenwerth *et al.*, 2003).

Statistical analysis

The PLFA profiles were analyzed using Sherlock PLFA tool (Version 1.1). The Shannon's diversity index or Shannon-Weaver index (*H*) was calculated: (-

$\Sigma p_i \ln p_i$), where p_i is the peak area of the i^{th} peak over the area of all peaks. Pielou's evenness index (*J*) was calculated as: (*H*/*Hmax*); where *H* is the no. derived from Shannon diversity index, and *Hmax* is the maximum value of *H* (*Hmax* = ln*R*; *R* = PLFA richness). Principal component analysis was performed using SPSS 18.0 software. Cluster analysis based on distance matrix revealed the relatedness based on the relative distribution of 59 PLFAs across the sites. Redundancy analysis (RDA) was performed using Microsoft Excel XLSTAT-2014 (Version 2.03).

Results and Discussion

Community level PLFA profiling have been found to be useful in detecting the responses of soil microbial communities to a varieties of land uses or disturbances in ecosystems (Yao *et al.,* 2000; Harris, 2003). Certain marker PLFAs can indicate relative amounts of certain functional groups of soil microorganisms (Zak *et al.,* 1994; Zelles, 1997). The qualitative and quantitative changes in microbial community structure not only determine the microbial diversity, but also the function and nature of interactions among the existing microbial species as well as the physiological state of the ecosystem. Besides, the existence of different functional groups responds differently to prevailing environmental conditions in different ecosystems, which influence the microbial community composition. There are useful biomarkers or signatures for fingerprinting the existence of soil microbial community because of the relative abundance of certain PLFAs, which differ considerably among the specific groups of soil microorganisms (Zelles *et al.,* 1994; Hill *et al.,* 2000; Kaur *et al.,* 2005; Lores *et al.,* 2010; Dickens *et al.,* 2013).

PLFA profiles of mine spoil samples

The relative contribution of 59 PLFAs representing microbial community structure across six different age series coal mine overburden spoil in chronosequence as well as the nearby forest soil showed marked differences (Table 1). Higher relative abundance of three fungal PLFAs [18:1ω9c (oleic acid), 18:2ω6c (linoleic acid), 18:3ω6c (gamma-linoleic acid)] (Olsson, 1999; Hill *et al.,* 2000; Myers *et al.,* 2001; Lores *et al.,* 2010; Dickens *et al.,* 2013) were observed in NF, which accounted for 4.19%, 1.48% and 0.97% respectively. PLFA 18:1ω9c (oleic acid) is reported to be most common in fungal species (Zelles, 1999). High prevalence of fungal PLFA in NF may be attributed to the availability of high amounts of recalcitrant polymeric phenolic compounds (lignin and tannin), their ability and principally responsible for lignin degradation (Cairney and Meharg, 2002). Similarly, higher abundance of arbuscular mycorrhizal fungal PLFA 16:1ω5c (*cis*-11-palmitoleic acid) were observed in NF (5.21%) as compared to different age series coal mine overburden spoil (Dickens *et al.,* 2013). PLFA 16:1ω5c derived from arbuscular mycorrhizal fungi are known to contribute substantially to the fungal biomass in NF (Olsson, 1999), which responds to changes in easily available C (Frostegard *et al.,* 1996; Hackl *et al.,* 2005). Highest relative abundance of methyl branched PLFAs 10Me18:0 (1.25%) and 10Me19:1ω7c (1.35%) were exhibited by OB_0 representing actinomycetes (Kroppenstedt, 1985; Zelles, 1999; Hill *et al.,* 2000). Besides, OB_8 and NF also exhibited 0.25% and 0.28% of actinomycetes PLFA 10Me20:0 respectively. Similarly, 0.14%

of actinomycetes PLFA 10Me19:1ω7c was exhibited by OB_2 (Table 1). However, the methanobacter PLFA 16:1ω8c (Hill *et al.,* 2000) was neither exhibited by the different age series coal mine overburden spoil nor by the nearby NF soil.

Distribution of PLFAs

The relative distribution of PLFAs in six different age series coal mine overburden spoil in chronosequence as well as nearby forest soil can be classified into distinct microbial community structure such as aerobic prokaryotes (monounsaturated fatty acids: MUFA), microeukaryotes (polyunsaturated fatty acids: PUFA), gram-positive and other anaerobic bacteria (saturated and branched fatty acids ranges from C_{14} to C_{16}), anaerobic bacteria (saturated and branched fatty acids ranges from C_{16} to C_{19}) (Morgan and Winstanley, 1997).

The PUFAs are considered to be the signature acids for eukaryote, which ranges from 35.01% (OB_8) to 57.09% (OB_0) (Table 2). The MUFAs representing aerobic prokaryotes can occur both in gram-negative and gram-positive bacteria that ranges from 3.36% (OB_6) to 19.68% (OB_{10}). However, their relative contribution to the total PLFA content in gram-positive bacteria is very small (*e.g.* < 20%), and thus MUFAs can be used as general biomarkers for gram-negative bacteria (Ratledge and Wilkinson, 1988). Higher level of unsaturated fatty acids with low levels of PUFAs supported the bacterial dominance.

Highest level of straight chain PLFAs was exhibited by OB_8 (46.47%) as compared to other soils. Branched chain PLFAs varies from 6.75% (OB_4) to 11.45% (OB_0). Branched-chain fatty acids have been used as biomarker for bacteria including anaerobic and sulfate-reducing bacteria. Branched-chain fatty acids (*iso* and *anteiso*) are characteristics of gram-positive bacteria, whereas *cyclopropyl* fatty acids are common in some gram-negative and anaerobic gram-positive bacteria (Ratledge and Wilkinson, 1988). The differences in the relative distribution of branched and MUFAs have been used as a marker for the proportion of gram-positive and gram-negative bacteria (Morgan and Winstanley, 1997).

The gram-negative bacteria contain unique hydroxyl fatty acids in lipid portion of lipopolysaccharides in cell wall, which is used as an indicator of gram-negative bacteria in environmental samples (Parker *et al.,* 1982; White, 1994). It is evident from the study that the soil microbial groups with hydroxyl fatty acids were confined to OB_0 (0.09%) and OB_2 (0.12%). Higher relative abundance of methyl branching PLFAs was observed in OB_0 (0.78%), as compared to different age series coal mine overburden spoil (Table 2). However, the distribution of MUFAs and PUFAs in NF accounted to 8.21% and 50.92% respectively (Table 2). Besides, highest level of PLFAs 18:1w9c (3.86%) and 18:2w6,9c (1.41%) representing fungi were observed in NF as compared to different age series coal mine overburden spoil.

The study indicated that the differences in PLFA profiles could be attributed to the variation in lipid contributing microbial communities and environmental conditions (Rajendran *et al.,* 1995; Claassens *et al.,* 2006), as well as development of microbial communities during the spontaneous succession on mine overburden spoil across the sites (Urbanova *et al.,* 2011).

Microbial community composition

PLFAs have several features that reinforce their use as indicator of environmental stress. They respond to environmental disturbances either by altering PLFA composition in microbial membrane (phenotypic plasticity) or shifting in soil microbial community structure (Kaur *et al.,* 2005). Marked differences in microbial community composition were observed across different age series coal mine overburden spoil profiles as well as forest soil (Table 3).

The fresh coal mine overburden spoil (OB_0) represents a disequllibriated geomorphic system with altered physico-chemical properties and the resultant biotic deficiency, which disrupt the 'geology-soil-plant' stability and the pedogenic processes (Claassens *et al.,* 2006; Urbanova *et al.,* 2011; Maharana and Patel 2013a). Higher levels of MUFA (Parkes and Taylor, 1983; Rajendran *et al.,* 1995) with lower level of PUFA were reported as the biomarker for gram-negative bacteria (Ratledge and Wilkinson, 1988) that explained the abundant distribution of gram-positive bacteria in OB_0 (14.58%). Besides, the presence of hydroxy PLFAs revealed the higher occurrence of gram-negative bacteria (Parker *et al.,* 1982; White, 1994) in OB_0 (27.18%) as compared to different age series coal mine overburden spoils as well as NF (Table 3). Further, higher level of gram-positive bacteria (14.58%) and anaerobes (4.77%) were also estimated in OB_0, which may be due to the higher occurrence of branched chain fatty acids (Parkes and Taylor, 1983), and abundantly distributed in anaerobic bacteria and gram-positive bacteria (Guckert *et al.,* 1985). The study revealed higher relative dominance of gram-negative bacterial PLFAs in metal contaminated soil (OB_0) with concomitant decrease in gram-positive bacterial PLFAs (Frostegard *et al.,* 1993; Zelles, 1994; Liao *et al.,* 2005) in chronosequence coal mine overburden spoil over time. Higher level of DMA PLFAs revealed the highest distribution of anaerobes (4.77%) (Frostegard *et al.,* 1991; Zelles, 1997; Zelles, 1999) in OB_0 as compared to different age series coal mine overburden spoil as well as NF. The methyl-branched PLFAs showed dominance of actinomycetes (Kroppenstedt, 1985; Zelles, 1999; Hill *et al.,* 2000) in OB_0 (0.99%), which may be due to their ability to withstand water stress (low water potential) by resisting plasmolysis and maintaining cell turgor by accumulating compatible solutes (proline and glycerol).

In addition, they are filamentous, enabling them to bridge air gaps between thin water films that occur in soil pore spaces during soil desiccation (Moore-Kucera and Dick, 2008). Lower fungal PLFAs (18:1ω9c, 18:2ω6,9c) suggested minimal fungal abundance in OB_0 (0.51%). However, it is evident from the data that OB_0 was found to be devoid of methanobacter population due to the absence of PLFA 16:1ω8c, which reflects the distribution of methanobacter (Hill *et al.,* 2000). Lower longer chain fatty acids in OB_0 (51.96%) indicated comparatively lower input from microeukaryotes (Smith *et al.,* 1986). The study indicated that the mode of action of heavy metals seems to interact with microbial membrane proteins resulting disturbances in protein conformations and activities (Frostegard *et al.,* 1993; Rajapaksha *et al.,* 2004; Liao *et al.,* 2005).

The ability of soil to maintain microbial community composition, nutrient concentration and functioning after a disturbance defines the resistance capacity

of a system. Resilience refers to the response of the system impacted by a disturbance, and can be defined as the rate of recovery in the original versus restored state of system. In addition to the abiotic factors, soil microbial community composition is considered as one of the major components of soil resilience due to their key role in nutrient cycling. Therefore, the microbial community composition in different age series coal mine overburden spoil in chronosequence over time should be compared with OB_0. Comparatively higher levels of MUFA (Parkes and Taylor, 1983; Rajendran *et al.*, 1995) with lower level of PUFA were observed in OB_2 with respect to OB_0, which explained the higher occurrence of gram-negative bacteria in OB_2 (22.23%). Besides, the relative dominance of hydroxy PLFAs in OB_2 revealed the higher occurrence of gram-negative bacteria (Parker *et al.*, 1982; White, 1994) in OB_2 as compared to different age series coal mine overburden spoils as well as NF (Table 3).

Besides, higher level of gram-positive bacteria in OB_2 (13.42%) may be due to the higher occurrence of branched chain fatty acids. Because of the lower occurrence of DMA PLFAs, lower level of anaerobes in OB_2 (3.44%) was observed (Table 3). Higher level of fungal PLFAs (18:1ω9c) revealed higher fungal dominance in OB_2 (0.82%) as compared to OB_0 due to the gradual establishment of vegetation, plant inputs of litter and exudates (Potthoff *et al.*, 2006; Yu and Ehnerfeld, 2010). The methyl-branched PLFAs reflect the actinomycetes (Kroppenstedt, 1985; Zelles, 1999; Hill *et al.*, 2000), which was found to be comparatively less in OB_2 (0.53%) as compared to OB_0. The distribution of methanobacter was not observed in OB_2 due to the absence of 10-methyl branched fatty acids and PLFA 16:1ω8c. Further, higher occurrence of long chain fatty acids and PUFA supported the higher level of distribution of microeukaryotes in OB_2 (59.58%) as compared to OB_0. Thus, the recovery of resource heterogeneity and pool sizes following restoration would indicate resilience of the system and variation in soil microbial community composition.

PLFA profiles suggested higher level of gram-negative bacteria in OB_4 (16.23%) than OB_6 (15.85%), which may be due to the higher level of MUFA in OB_4 (Parkes and Taylor, 1983; Ratledge and Wilkinson, 1988; Rajendran *et al.*, 1995). Similarly, higher relative distribution of gram-negative bacteria was observed in OB_8 (16.67%) as compared to OB_{10} (14.92%). However, higher level of gram-positive bacteria was observed in OB_6 (13.81%) as compared to OB_4 (10.11%), which may be due to the higher occurrence of branched chain fatty acids in OB_6. Similarly, higher level of gram-positive bacteria was observed in OB_8 (13.12%) as compared to OB_{10} (11.98%). Lower level of anaerobes was estimated in OB_6 (3.48%) as compared to OB_4 (3.99%), which may be due to the lower occurrence of DMA PLFAs. Similarly, OB_{10} (3.57%) exhibited lower level of anaerobes as compared to OB_8 (3.87%). The distribution of actinomycetes was not observed in OB_4 and OB_6 due to the absence of the methyl-branched PLFAs. However, the methyl-branched PLFAs representing actinomycetes (Kroppenstedt, 1985; Zelles, 1999; Hill *et al.*, 2000) was found to be higher in OB_8 (0.33%), which was found to be higher as compared to OB_{10} (0.15%). Higher level of fungal PLFAs (18:1ω9c) revealed higher fungal dominance in OB_{10} (0.63%) as compared

to OB_8 (0.59%). Similarly, higher relative distribution of PLFA 16:1ω5c reflects the dominance of arbuscular mycorrhizal fungi in OB_{10} (0.52%) as compared to OB_8 (0.25%). Further, higher longer chain fatty acids indicated comparatively higher input from microeukaryotes in OB_{10} (68.25%) as compared to OB_8 (65.18%)

The level of distribution of gram-positive, gram-negative bacteria and anaerobes in nearby NF was found to be 9.48%, 12.93% and 3.57% respectively (Table 3). Higher relative abundance of gram-negative as compared to gram-positive bacteria in NF indicating the profound effects of plants have on soil development and lipid profiles. Highest abundance of arbuscular mycorrhizal fungi (2.77%), fungi (1.25%) and microeukaryotes (69.56%) were observed in NF, which may be due to the greater litter inputs and root turnover, and symbiotic nitrogen fixation contributed to the formation of highly localized soil resources characterized by higher concentrations of C and N that are believed to support more diverse population of heterotrophic soil microorganisms. Further, fungi are uniquely adapted to degrade substrate (lignin), and formation of organic matter (Cairney and Meharg, 2002). Comparative analysis of the level of distribution of PLFAs across the sites suggested that the heavy metal contamination in mine overburden spoil (Frostegard *et al.,* 1993; Pennanen *et al.,* 1996; Zelles, 1999; Rajapaksha *et al.,* 2004; Liao *et al.,* 2005) resulted a decrease in PLFAs (a15:0, 16:1ω5c, 18:1ω7c, 18:1ω9c, 18:2ω6c, 18:3ω6c) in OB_0 as compared to undisturbed nearby NF soil.

Further, the PLFA markers used to quantify the relative abundance of specific gram-positive to gram-negative bacteria ratio in different age series coal mine overburden spoil as well as the nearby NF soil were as follows: i14:0, i15:0, a15:0, i16:0, 10Me16:0, i17:0, a17:0, 10Me17:0 for gram-positive bacteria, and 15:1ω4c, 16:1ω7c, 16:1ω9c, cy17:0, 17:1ω9c, 18:1ω7c, 18:1ω9c, cy19:0; cy19:0ω7c for gram-negative bacteria (Frostegard *et al.,* 1996; White *et al.,* 1996; Zelles, 1997; Fierer *et al.,* 2003). The analysis revealed that the mean ratio of gram-positive to gram-negative significantly decreased from OB_0 (7.478) to OB_{10} (1.795), and was found to be minimum in NF (1.250) as compared to different age series coal mine overburden spoil (Figure 2).

This was mainly attributed to the increase in gram-negative bacteria, thus suggesting that the gradual improvement in organic carbon in a chronosequence coal mine overburden spoil due to vegetation development over time (Maharana and Patel, 2013b) provided a more stable and readily available substrate for supporting higher levels of microbial activity of gram-negative bacteria (Peacock *et al.,* 2001). Several investigators have reported that the gram-negative bacteria were mainly associated with monounsaturated fatty acids (MUFA), which corresponds to the gradual increase in organic matter content and high substrate availability (Bossio and Scow, 1998; Zelles *et al.,* 1992).

Thus, the study indicated that the combined effects of changes in both aboveground and belowground inputs would influence soil microbial community by affecting the C inputs from root exudates and litter (Myers *et al.,* 2001) in a chronosequence coal mine overburden spoil in course of time suggesting the process of restoration (Claassens *et al.,* 2006; Urbanova *et al.,* 2011).

Shannon-Weaver diversity index

The ability of an ecosystem to withstand extreme disturbances may contribute to microbial community structure and hence microbial diversity. Diversity index is a quantitative measure, which not only accounts for the existence of different PLFAs richness (*R*), but also accounts how evenly they are distributed (evenness). The Shannon-Weaver index (*H*) has been a popular diversity index frequently used in microbial ecology studies. The bacterial and fungal PLFAs are used as a measure of the relative distribution of different microbial groups (fingerprints of soil microbial community) because of the relative abundance of certain PLFAs (Bardgett and McAlister, 1999), which differ considerably among different microbial groups (Zelles *et al.,* 1994). The study revealed a significant variation in PLFA richness, Shannon diversity index, and evenness across different age series coal mine overburden spoil in chronosequence as well as nearby NF soil (Table 4). Greater PLFA richness (*R*) was attributed by OB_8 (45) as compared to other soil profiles. The Shannon diversity index (*H*) across the sites varies from 2.2629 (OB_6) to 2.8385 (OB_8).

Besides, the evenness is defined as a measure of diversity index, which quantifies how equal the community is numerically. The evenness of a community represented by Pielou's evenness index (*J*) is constrained between 0 and 1. The evenness of PLFA reflects the broad-scale changes in terms of the relative dominance of certain microbial groups (Kaur *et al.*, 2005). The Pielou's evenness index (*J*) based on the distribution of 59 PLFAs across the sites varies from 0.7120 (OB_6) to 0.7952 (OB_4) (Table 4). The more even the distribution of PLFAs or less variation in community between microbial groups, greater is the diversity. Thus, the value of diversity index increases when both the number of types of PLFAs and evenness increases.

Further, the Shannon diversity index based on the distribution of different microbial groups with respect to different coal mine overburden spoil as well as nearby forest soil was calculated. Higher Shannon-Weaver index in OB_2 (1.0676) suggesting higher population diversity as compared to OB_0 (0.6417). Similarly, higher level of microbial diversity was exhibited by OB_6 (0.9827) as compared to OB_4 (0.9611). Further, OB_{10} (0.8579) exhibited lower microbial diversity than OB_8 (1.0340), which indicated that the microbial communities in less disturbed ecosystems like OB_{10} may be dynamic in terms of functional responses to a perturbation but more resistance to changes in community composition (Steenwerth *et al.,* 2003). Besides, the differences in microbial community structure and associated diversity among different age series coal mine overburden spoil may be attributed to the variation in microbial biomass nutrient to soil nutrients ratio (MB-C:OC), which represents the quantum of soil nutrients reflected in the microbial biomass, and functional index of the soil subsystem (Insam and Domsch, 1988).

Fungal:bacterial biomass ratio

The fundamental differences in bacterial and fungal physiology and ecology would suggest that the biogeography of each group would be controlled by separate edaphic factors, which may vary among different soil profiles (Van dar Wal *et al.*, 2006). As the bacteria and fungi are likely to have distinct functional roles in different soil profiles, a more robust

understanding of the specific effects of land-use and edaphic factors on these two microbial groups will improve our ability to predict the specific effects of land-use changes on soil microbial community structure and function (Claassens *et al.,* 2006).

The fungal biomass was calculated based on the relative distribution PLFA 18:2ω6c across the sites. Similarly, the total bacterial biomass was obtained by summation of the distribution of PLFAs 14:0, 15:0, a15:0, i15:0, i16:0, 16:1ω7c, 16:1ω11c, 10Me 16:0, 17:0, a17:0, cy17:0, i17:0, 17:1ω8c, 10Me 17:0, 18:0 2OH, 18:1ω5c, 18:1ω7c, 10Me 18:0, 19:1ω6c and cy19:0ω8c (Fraterrigo *et al.,* 2006). An index of fungal to bacterial (F/B) ratio of the microbial biomass was used to study the state of soil microbial community in response to different environmental stresses (Kaur *et al.,* 2005). The F:B ratio was reported to be a potential tool to discriminate the disturbed from undisturbed soil system (Bradgett and McAlister, 1999; Bailey *et al.,* 2002; Claassens *et al.,* 2006; Moore-Kucera and Dick, 2008).

The F:B ratio exhibited an increasing trend from OB_0 (0.0216) to OB_{10} (0.0990). Comparatively higher F:B ratio was estimated in OB_4 (0.0307) as compared to OB_2 (0.0277). In addition, OB_8 (0.0757) exhibited higher F:B ratio as compared to OB_6 (0.0649). However, the difference in F:B ratio in chronosequence mine overburden spoil was less pronounced due to extreme environmental conditions as well as heavy metal contamination (Frostegard *et al.,* 1993; Pennanen *et al.,* 1996; Zelles, 1999; Liao *et al.,* 2005). Highest F:B ratio was observed in NF (0.1128) as compared to different mine overburden spoil, which may be due to the higher prevalence of fungal PLFAs exhibiting higher C:N ratio and low bulk density (Maharana and Patel, 2013a). The capacity of fungi for translocation N to C sources is thought to be important in NF with high C:N ratio (Bailey *et al.,* 2002; Bardgett and McAlister, 1999). In accordance with multiple surveys of fungal to bacterial ratio, it was observed that the F:B ratio was higher in forest soil (Bailey *et al.,* 2002; Fierer *et al.,* 2005; Hogberg *et al.,* 2007). Besides, NF appeared to be set apart from other soil profiles by a higher abundance of arbuscular mycorrhizal fungi (2.77%), which may be better able to cope with available N and organic matter. Higher F:B ratio in NF can be explained on the basis of the existence of higher relative distribution fungal PLFAs (1.25%) as compared to coal mine overburden spoil.

In addition, NF was supported with distinct microbial communities that are correlated with factors that define the land-use history and the associated soil quality influence microbial community composition (Steenwerth *et al.,* 2003; Claassens *et al.,* 2006). The study indicated that the disturbed ecosystems have lower F:B ratio (Bradgett *et al.,* 2001), whereas the organically managed soil systems have increased F:B ratio than conventional system (Bradgett *et al.,* 1997).

Further, the change in microbial community structure inhabiting in different landscape caused by spatial variability in soil pH, moisture content, nutrient availability could affect microbial transformations altering nutrient cycling processes, which will be useful in providing insight how these microbes could affect the fertility status of soil environment. Differences in soil pH can

arise due to variation in vegetation type, soil type and management regime. Thus, soil pH may serve as a convenient integrating variable representing the physico-chemical characteristics of a particular soil and reasonably a good predictor of microbial community composition (Fierer and Jackson, 2006; Lauber *et al.,* 2008). The decline in soil pH from NF to OB_0 (Maharana and Patel, 2013a) may be one of the major constraints/stress shifting microbial community structure under low soil pH (Hackl *et al.,* 2005). Comparative analysis of F:B ratio suggested that lower pH (Bath and Anderson, 2003) resulted a decrease in PLFAs (a15:0, 16:1ω5c, 18:1ω7c, 18:1ω9c, 18:2ω6c, 18:3ω6c) in OB_0 as compared to undisturbed NF soil.

The decreased stress with gradual improvement in soil pH towards neutral in NF soil (Maharana and Patel, 2013a) could be related to an increase in F:B ratio (Baath and Anderson, 2003) due to the nutrient availability leading the shifting in microbial community structure (Persson *et al.,* 1989; Baath *et al.,* 1995; Merila *et al.,* 2002; Claassens *et al.,* 2006; Urbanova *et al.,* 2011) across a chronosequence coal mine overburden spoil. The correlation between F:B ratio and soil pH was analyzed to be statistically significant ($r = 0.966$, $p < 0.001$), which suggested that soil pH can account 93.47% of the variability in F:B ratio (Figure 3a). Besides, the gradual increase in moisture from OB_0 to NF soil (Maharana and Patel, 2013a) may also affect soil microbial community as well as F:B ratio through its effect on osmotic potential, transport of nutrients and energy, and cellular metabolism as well as on the competitive interactions between microbial species (William and Rice, 2007; Meimei *et al.,* 2008). Soil moisture content exhibited a positive correlation with F:B ratio ($r = 0.784$, $p < 0.001$), which can account 61.48% of the variability in F:B ratio across the sites (Figure 3b). Changes on soil moisture can affect the composition and function of soil microbial community due to differences in drought tolerance among taxonomic and functional groups of microorganisms (Gray *et al.,* 2011; Zhou *et al.,* 2014).

Cluster analysis

Relative distributions of 59 PLFAs among different coal mine spoil as well as nearby forest soil profiles were subjected to cluster analysis based on the distance matrix revealed the existence of six clusters (I – VI) in the dendrogram (Figure 4). The analysis revealed highest similarity (53.4372) between OB_2 and OB_4 (cluster-VI). The relatedness between OB_0 and OB_6 (cluster-V), and OB_2 and OB_{10} (cluster-IV) exhibited similarity level 44.8328 and 44.3818 respectively. The similarity level between OB_2 and NF was estimated to be 43.5123 (cluster-III). OB_0 and OB_2 exhibited similarity level (40.7281) representing cluster-II. Minimal similarity level (32.7009) was observed between OB_0 and OB_8 (cluster-I). The study indicated that the six clusters based on the relative distribution of 59 PLFAs exhibited the tree likeness of original (unrandomized) tree was statistically well resolved.

Further, in order to discriminate six different age series coal mine overburden spoil as well as NF soil profiles, principal component analysis was performed (Ludwig and Reynolds, 1988) on the basis of the relative distribution of 59 PLFAs among the microbial communities. The eigen vectors determine the direction of maximum variability, and the eigen values

specify the variances. The principal component analysis suggested that the Z1 and Z2 components explained the maximum variance with their cumulative percentage of variance estimated to be 49.9%. The relative distribution of 59 PLFAs revealed differential microbial community structure among six different coal mine overburden spoil in chronosequence as well as the nearby forest soil profiles, which were well segregated (Figure 5).

Multivariate analyses

The redundancy analysis (RDA) can able to examine the relationships between different soil profiles, species, and environmental gradients altogether not only in concert in the same model, but also unlike discriminate analysis there is no limit on the number of species that can be used relative to the number of samples. Changes in soil microbial community structure may occur in response to altered soil physico-chemical properties that affect the soil microenvironment with possible effects on the efficiency of readily mineralizable resource conservation by soil microbes.

RDA analysis allowed examining the variation in PLFA patterns in terms of both mine overburden sites and the measured environmental gradients including enzyme activities, which was found to be significant ($p < 0.005$). A total of 58.05% of the variation could be explained based on the fitted PLFA data by the model from the canonical sum of the eigen values. The six different age series coal mine overburden sites and the environmental gradient arrows including enzyme activities for the RDA ordination of the PLFA data were shown (Figure 6a). The slit and clay %, moisture content (MC), water holding capacity (WHC), soil pH, organic C (OC), total N (TN), extractable P (EP) as well as enzyme activity (amylase, invertase, protease, urease, phosphatase and dehydrogenase) increased in the general direction of OB_8 and OB_{10}, while sand % and bulk density (BD) increased towards OB_0. This study provides an insight into the multifaceted nature of these factors that shape the microbial community structure in chronosequence coal mine overburden spoil (Claassens *et al.*, 2006; Urbanova *et al.,* 2011).

The data related to the physico-chemical properties (Maharana and Patel, 2013a) as well as enzyme activities (Maharana and Patel, 2013b) in chronosequence coal mine overburden spoil were taken from our earlier investigation for RDA analysis. The proportions of certain PLFAs were highly correlated with different soil physico-chemical properties (Claassens *et al.,* 2006; Urbanova *et al.,* 2011) in different age series coal mine overburden spoil over time (Figure 6b). The clay, pH, MC, WHC, OC, TN, EP and the enzyme activities were highly correlated with PLFAs (a14:0, 14:1ω7c DMA, 14:1ω8c, 16:1ω5c, 16:1ω7c, 18:1ω7c, 18:1ω9c, 18:2ω6c, 18:3ω6c, 21:1ω8c and 24:0), while sand and BD with PLFAs (12:1ω8c, 14:1ω5c, a15:0, 16:0 aldehyde, 16:0 2OH, 16:2 DMA, 10Me 18:0, 18:1ω7c DMA, 19:0cy ω7c and 10Me 19:1ω7c) in different age series coal mine overburden spoil.

Further, the negative correlation coefficients indicated that changes in microbial community structure in response to soil disturbances were associated with a decrease in the respective soil properties among different soil profiles. Although all 59 PLFAs were included in the RDA

ordination, for the shake of clarity the PLFAs with the highest species scores on each of the first two ordination axes, those that correlated well with the environmental variables and important biological markers are displayed (Figure 6b).

Some general patterns emerge from this analysis. The existence of higher level of methyl-branched PLFAs (10Me18:0; 10Me 19:1ω7c), and saturated branched fatty acids (C_{16} to C_{19}) in OB_0 revealed that higher relative abundance of actinomycetes, anaerobic bacteria respectively. In addition, the PLFA a17:0 suggested the higher distribution of sulfate reducing bacteria, which may be due to the pyrite (FeS_2) contamination in OB_0. Further, the level of saturated branched fatty acids (C_{14} to C_{16}) was found to be comparatively higher in OB_{10} as compared to different age series coal mine overburden spoil suggesting the higher relative abundance of gram-positive bacteria in OB_{10}. Minimal longer chain PLFAs in OB_0 indicated comparatively lower input from microeukaryotes, which may be influenced by acidic pH and metal induced toxicity.

However, highest relative abundance of arbuscular mycorrhizal fungi (16:1ω5c) and heterotrophic microeukaryotes were observed in OB_{10}. Higher level of fungal PLFAs 18:3ω6c, and PLFAs 18:1ω9c, 18:2ω6c suggested higher relative distribution of fungal population in OB_{10} and OB_8 respectively.

Besides, higher level of PLFAs 16:1ω7c and 18:1ω7c suggested higher relative distribution of aerobic bacteria in OB_{10} and OB_8 respectively. The study suggested that the shift in microbial community structure from OB_0 to OB_{10} in course of time may be attributed to the gradual change in soil quality in the direction of OB_{10} supplementing the process of reclamation.

The most reliable manner to measure changes in an ecosystem, and understanding the ecosystem structure and function is through long-term monitoring employing appropriate spatial and temporal scales. A realistic ecological assessment of mine spoil reclamation implies monitoring the site through time. However, the inherent difficulties associated with the monitoring of restoration processes combined with the fact that it can't be replicated using traditional experimental approaches, which have necessitated the use of alternative monitoring approach through time to quantitatively assess the reclamation process.

PLFA analysis indicated physiological stress and can be used to compare the physiological status of microbial communities in different soil profiles. The multivariate analysis revealed that six different age series coal mine overburden spoil had distinctly different PLFAs, and microbial community composition. Changes in microbial community structure may occur in response to altered soil physico-chemical properties with possible effects on the efficiency of C conservation by the microorganisms.

Nevertheless, the readily mineralizable source of organic matter would enhance the responses of soil microbial processes including enzyme activities to change the soil microenvironment. Thus, PLFA profiling provides a sensitive and meaningful measure of microbial community composition to monitor ecosystem restoration based on the soil quality assessment in a chronosequence coal mine overburden spoil compared with the nearby undisturbed NF soil.

Table.1 Percentage composition of 59 PLFAs in coal mine overburden spoil profiles as well as nearby forest soil

PLFAs	OB_0	OB_2	OB_4	OB_6	OB_8	OB_{10}	NF
12:0	15.67	18.35	14.43	24.55	15.95	14.29	16.43
12:1ω8c	0.6	0	0	0	0	0.24	0
a13:0	1.91	1.61	1.04	2.61	1.53	0.73	2.05
13:1ω5c	0.75	0.38	0.69	0	0.58	0.38	0.63
14:0	0.38	0.57	1.28	0	0.12	0.61	0.49
a14:0	0	0	0	0	0.11	0.18	0.23
i14:0	0	0	0	0	0.17	0	0.31
14:1 ω5c	0.57	0.48	0	0.6	0.05	0	0.48
14:1 ω7c DMA	0	0	0.49	0	0.49	2.52	0
14:1ω8c	0	0	0	0	0.15	0.24	0.24
15:0	0	0	0	0	0.48	0	0
a15:0	3.24	2.68	2.77	2.99	2.28	2.59	3.29
i15:1ω6c	0.48	0.64	0.56	0.79	0.53	0.57	0.7
15:1ω8c	0	0.44	0.58	0	0	0	0
15:1ω9c	0	0	0	0	0.09	0	0
a15:1ω9c	0.3	0.25	0	0	0	0.26	0
15:3ω3c	24.23	15.61	16.66	24.33	14.06	14.84	17.56
16:0	5.74	10.94	13.59	4.7	7.05	4.29	5.25
16:0 aldehyde	0.28	0.51	0	0	0	0	0.45
a16:0	0.63	0.2	0	0	0.18	0.34	0.36
16:0 N alcohol	0	0	1.22	0.52	0	0	0
16:0 2OH	0.13	0.12	0	0	0	0	0
16:1ω5c	0	0	0	0.34	1.14	1.44	5.21
16:1ω6c	0	0	0	0	0	0.11	0
16:1ω7c	0	0	0	0	0.08	0.33	0.27
16:1ω7c DMA	1.68	9.97	6.11	1.89	3.65	15.14	4.48
16:1ω9c aldehyde	0.19	0	0	0	0	0.26	0
16:2 DMA	4.32	1.95	2.11	2.36	1.42	0	0.75
16:4 ω3c	0	0	0	0	0.59	0	0
17:0	0.21	0.32	0	0.42	2.09	0	0
a17:0	3.64	2.47	2.59	2.5	1.9	3.37	2.84
17:1ω4c	0.5	0.32	0	0	0.27	0.43	0.35
a17:1ω7c	0	1.11	0	0	0	0	0.82
17:1ω7c	0.7	0.77	0.39	0	0.45	0.73	0.62
i17:1ω9c	0.61	1.46	0	0.43	0.84	2.09	1.36
18:0	2.91	5.12	6.43	2.73	10.45	2.1	2.41
10 Me 18:0	1.25	0.3	0	0	0.25	0	0.28
18:0 DMA	0	0.43	0.57	0	0	0	0
18:1ω7c	0.35	0.38	0.61	0.47	0.54	0.62	0.69
18:1ω7c DMA	2.21	0.59	0	0	0	0	0
18:1ω9c	0.57	1.17	1.73	1.84	2.02	2.37	4.19
18:2ω6c	0.32	0.49	0.64	0.72	1.12	1.17	1.48
18:3ω6c	0	0	0	0.25	0.58	0.64	0.97

19:0cy ω7c	1.26	1.12	0.53	0.34	0.16	0	0
10Me 19:1ω7c	1.35	0.14	0	0	0	0	0
19:1ω8c	0.36	0.18	0.4	0	0.22	0.29	0
19:3ω3c	15.9	9.01	12.93	13.44	8.58	10.76	12.22
19:4ω6c	0.33	0	0.64	0	0.26	0.71	0.31
20:0	0	0.16	0	0	9.53	0.25	0.17
20:1ω9c	0.29	0	0	0	0.09	0.16	0.22
20:2ω6c	0	0	0	0.21	0	0	0
21:1ω8c	0	0	0	0	0.09	0.18	0
21:3ω3c	5.89	6.12	9.83	10.65	5.65	9.04	8.05
22:0	0	0.13	0	0	0.14	0.14	0.19
22:1ω6c	0	0	0	0	0	0.15	0
22:1ω9c	0.29	0	0	0	0.14	0	0.25
23:3ω3c	0	3.53	0	0	3.45	4.21	3.04
23:4ω6c	0	0	1.17	0.28	0.35	0.57	0.43
24:0	0	0	0	0.21	0.24	0.57	0

Table.2 Distribution of different PLFAs (%) in different coal mine overburden spoil profiles as well as nearby forest soil

Sample	Straight	Branched	Hydroxy	MUFA	PUFA	DMA	18:1 w9c	18:2 w6,9c	10-methyl
OB_0	21.75	11.45	0.09	4.72	57.09	3.26	0.62	0.4	0.78
OB_2	32.99	9.99	0.12	13.35	39.24	2.27	1.28	0.53	0.35
OB_4	33.2	6.75	nd	8.95	45.85	2.66	1.89	0.71	nd
OB_6	27.67	9.99	nd	3.36	55.20	2.51	0.87	0.39	nd
OB_8	46.47	7.04	nd	8.41	35.01	2.08	0.51	0.21	0.28
OB_{10}	18.57	9.76	nd	19.68	47.44	2.91	1.01	0.51	0.13
NF	21.12	11.48	nd	8.21	50.92	2.71	3.86	1.41	0.32

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Table.3 Relative distribution of microbial community (%) across different coal mine overburden spoil profiles as well as nearby forest soil

Sample	Gram positive	Gram negative	Anaerobes	Actino-mycetes	A M Fungi	Fungi	Methanobacter	Eukaryote
OB_0	14.58	27.18	4.77	0.99	nd	0.51	nd	51.96
OB_2	13.42	22.23	3.44	0.53	nd	0.82	nd	59.58
OB_4	10.11	16.23	3.99	nd	nd	1.04	nd	68.64
OB_6	13.81	15.85	3.48	nd	nd	0.54	nd	66.32
OB_8	13.12	16.67	3.87	0.33	0.25	0.59	nd	65.18
OB_{10}	11.98	14.92	3.57	0.15	0.52	0.63	nd	68.25
NF	9.48	12.93	3.57	0.43	2.77	1.25	nd	69.56

Figure.2 Gram-positive to Gram-negative bacteria ratio in different age series coal mine overburden spoil as well as nearby NF soil

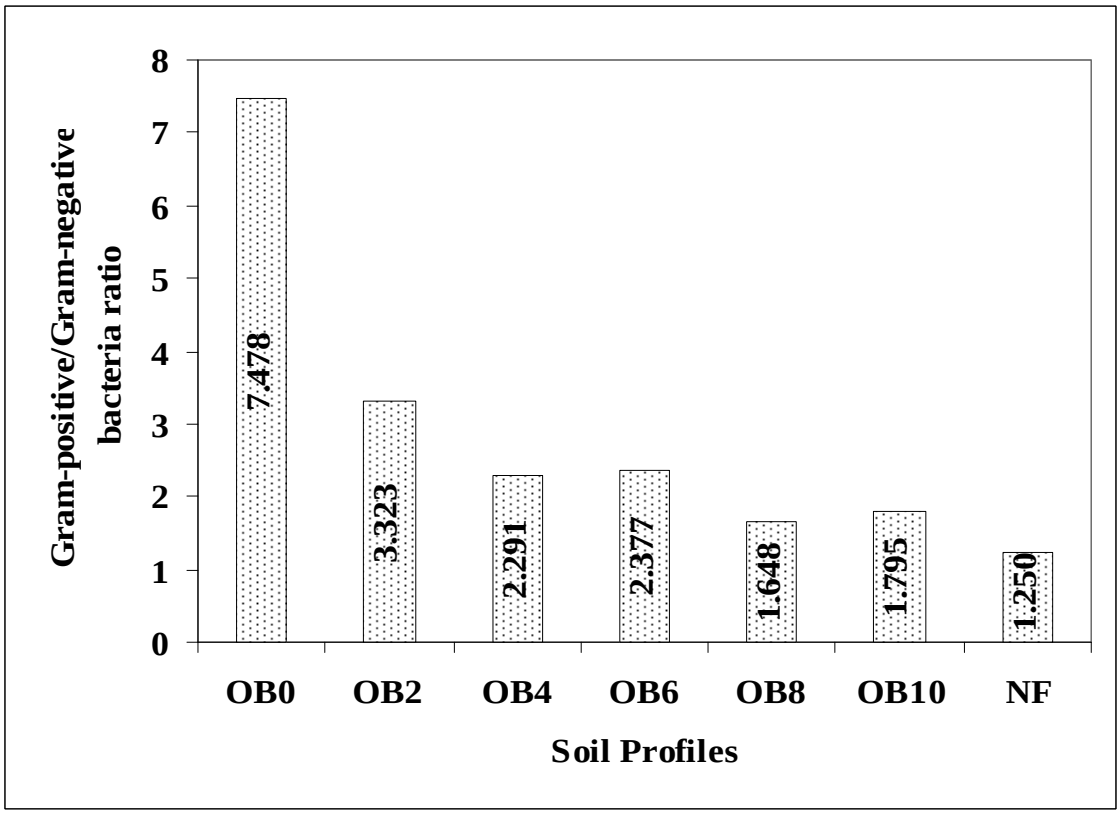


Table.4 Shannon diversity index and Pielou's evenness index based on the distribution of 59 PLFAs across different mine spoil profiles as well as nearby forest soil

Site	PLFA richness (*R*)	Shannon diversity index (*H*)	Pielou's evenness index (*J*)
OB_0	35	2.588892599	0.728168538
OB_2	36	2.682135617	0.748464195
OB_4	25	2.559730487	0.795224987
OB_6	24	2.262944128	0.712053429
OB_8	45	2.838519863	0.745671535
OB_{10}	40	2.731600405	0.74049598
NF	38	2.618224557	0.719769771

Figure.3 Correlation between fungal:bacterial ratio and (a) soil pH, and (b) moisture content in different age series coal mine overburden spoil as well as NF soil

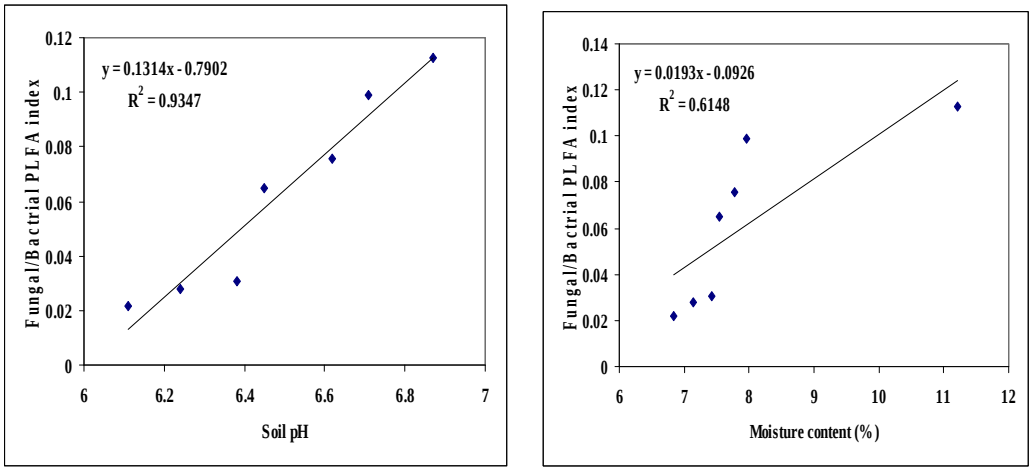


Figure.4 Cluster analysis illustrating the relatedness based on the relative distribution of 59 PLFAs among different age series mine overburden spoil as well as NF soil

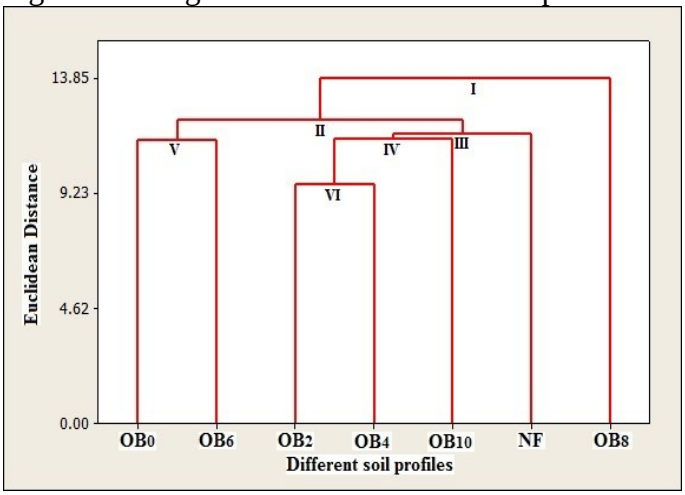
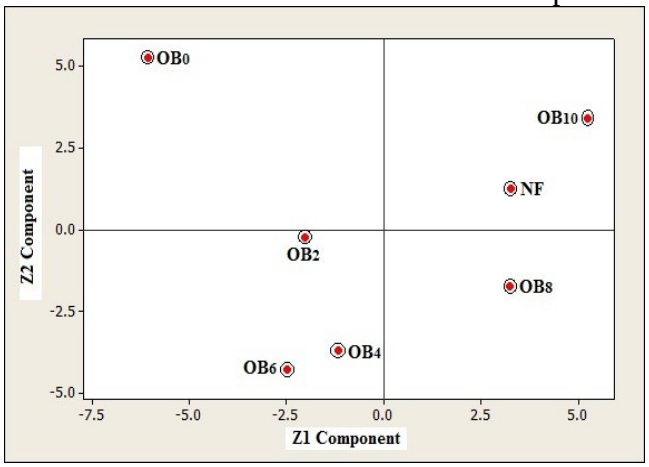
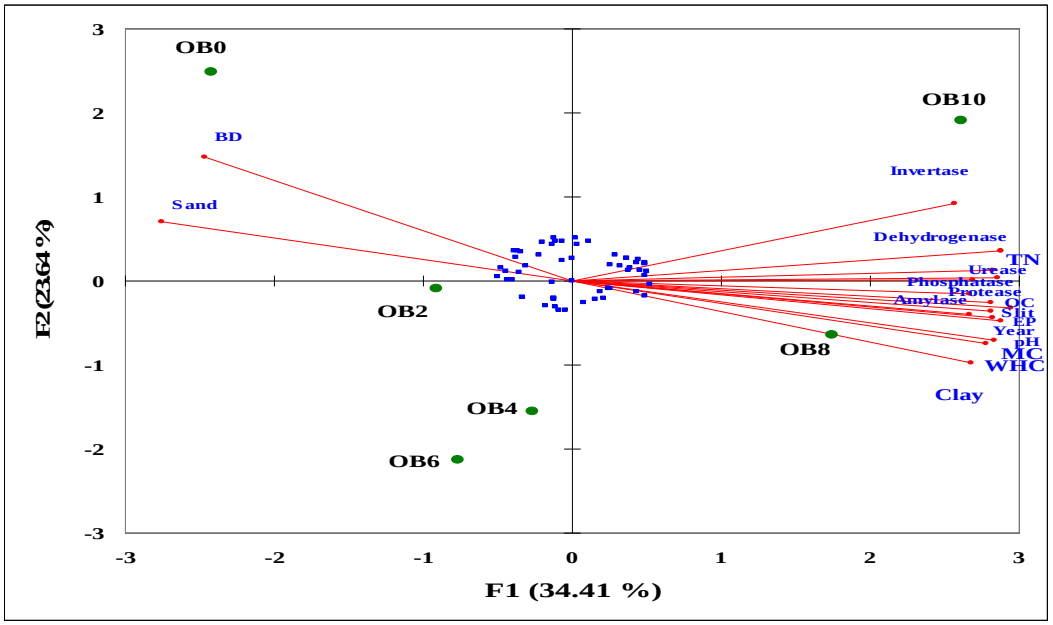


Figure.5 Principal component analysis based on the relative distribution of 59 PLFAs among the microbial communities in different mine overburden spoil as well as NF soil





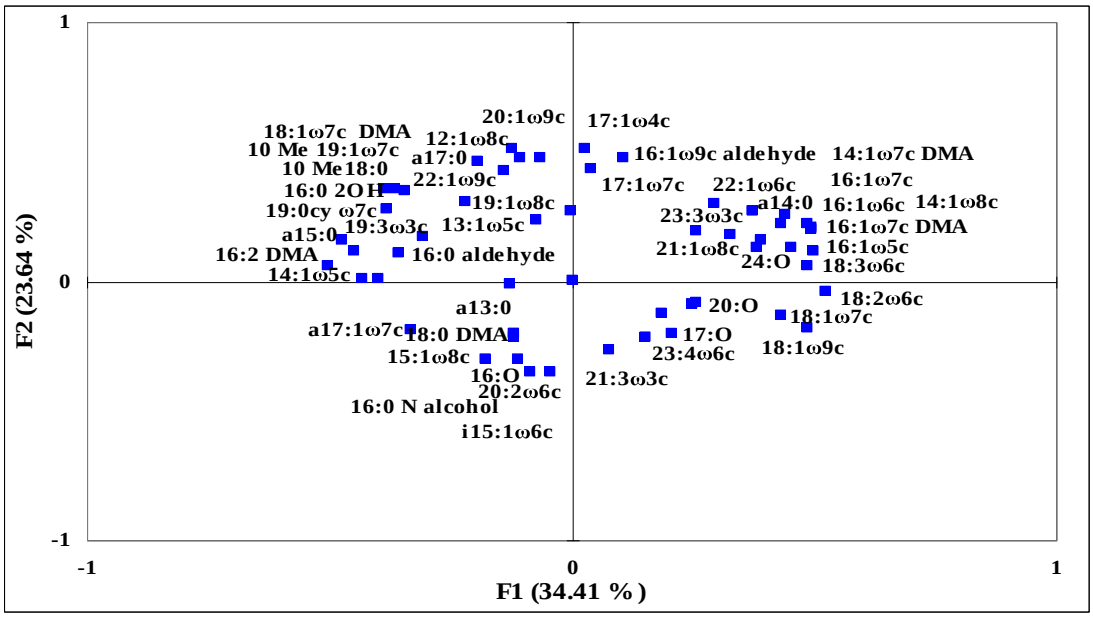


Figure.6 Redundancy analysis (RDA) of the PLFA data set for six different age series coal mine overburden spoil, using 59 PLFAs and 11 environmental variables. (a) site codes for each sample; (b) showed the PLFAs that had the highest absolute species scores on each of the first two axes, along with additional PLFAs of biological interest

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