Journal of Ecophysiology and Occupational Health, Vol 22(2), DOI: 10.18311/jeoh/2022/29162, 77-87, June 2022
ISSN (Print): 0972-4397
ISSN (Online): 0974-0805

PLFA Profiling of Coal Mine Spoil: An Integrated Approach for the Assessment of Ecological Restoration

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Abstract

Mine spoil generated after math of extensive coal mining activities represents disequilibrated geomorphic system. The pedodiversity including its link with biodiversity and landscape ecology describe the spatial diversity has emerged as functional determinants of ecosystem processes. Being the driving force in mediating soil processes, ecosystem restoration through mine spoil genesis is addressed to determine the shift in microbial communities in coal mine spoil over time. PLFA is independent of culture techniques that involve various molecular markers to determine microbial community structure as well as their discrimination based on their origin. PLFAs are rapidly degraded after cell lyses that are synthesized during microbial growth reliably reflecting the viable microbial communities. The study revealed significant variation with respect to their relative distribution of 51 PLFAs indicating the variations in microbial community composition with Shannon diversity index varies from 1.5265 (OB0) to 2.0139 (OB15) and Pielous evenness index from 0.4110 (OB0) to 0.5260 (OB15). Increasing trend in fungal to bacterial ratio was evident from OB0 (0.055) to OB15 (0.348) with the increase in age of mine spoil that revealed the progress in ecological restoration. Besides, the PCA and RDA analysis discriminate chronosequence coal mine spoil into independent clusters, which revealed the microbial community dynamics influencing the pace and progress of ecological restoration.

Keywords: Coal Mine Spoil, Microbial Community Structure, Mine Spoil Genesis, PLFA

1. Introduction

Ecological restoration through mine spoil genesis is dogmatic involving strategies that reflects a holistic approach[1,2], which not only involves periodic assessment using reliable soil quality indicators, but also ecological restoration of degraded ecosystem to self-sustaining ecosystem. The concept of pedodiversity describes spatial diversity associated with biodiversity and landscape ecology is considered as functional determinants of ecological restoration. Criteria for mine spoil genesis largely focus on microbial ecology of soil subsystems due to the fact that the vegetation patterns observed above ground are being driven by microbial diversity and soil processes[3]. Microbiological study provides the quantum of microbial pool size associated with their activities that cannot be considered as biomarkers due to redundancy of functions along with their interactions within microbial communities. Microbial community structure mediates organic matter decomposition and nutrient turnover at it regulates its pool size and activity[3]. Total microbial pool size remains constant even though changes in microbial community composition, which reflects the role of microbial communities4. Besides, microbial community structure is used for the assessment of biodiversity that can respond much faster to disturbances[2].

Being the difficulties in culturing microbes, the culture-independent approach is used to determine microbial community structure depending upon the existence of PLFAs in microbial membranes[5–7], which not only used to determine viable microbial biomass[8] but also nutritional/physiological status of different soil profiles[2,9]. PLFAs are signature molecules exclusively found in microbial membranes following rapid metabolism after cell lysis, exhibit high turnover rate and absent in storage lipid/anthropogenic contaminants[9]. PLFA profiling provides molecular markers for different microbial taxa, which can be used as sensitive biomarker of microbial stress for environmental assessment[10]. PLFA profiling can be used as robust tool involved in discriminating microbial communities based on their origin as well as soil management

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practices. PLFA profiling reflects broad diversity measures for rapid characterization of taxonomic groups of microbial community structure at phenotypic level[7,11,12]. Being the variation with respect to their PLFA patterns with varying chain length, branching and saturation exhibited by different microbial communities, the PLFA profiling can be used as 'microbial community fingerprint' of soil subsystems[9,13,14].

PLFA profiling can be used to categorize different microbial communities including micro-eukaryotes (PUFA), aerobic prokaryotes (MUFA), gram-positive and anaerobic bacteria with saturated and branched fatty acids (C_{14} to C_{16}), and branched-chain fatty acids with *iso* and *anteiso* PLFAs reflecting the occurrence of gram-positive bacteria. Besides, the Gram-negative bacteria revealed the possession of hydroxy PLFAs in lipid moiety of lipopolysaccharides present in their cell wall[15]. Sum of PLFAs (14:0, 15:0, 16:0, 17:0, 18:0, 18:1ω9c, 20:0, 21:0, 22:0, 24:0) is derived primarily from bacterial origin[9,13]. PLFAs such as 16:1ω7c, cy17:0, 18:1ω7c cy19:0 are prevalent in gram-negative bacteria[16–18], the *iso* and *anteiso* PLFAs such as a13:0, a14:0, i14:0, a15:0, i15:1ω6c, a15:1ω9c, a16:0, a17:0, a17:1ω7c, i17:1ω9c represent gram-positive bacteria in different soil profiles[16–18]. The sulfate-reducing bacteria including anaerobes are represented by saturated and branched (C_{16} to C_{19}) fatty acids[19]. PLFAs (18:1ω9c, 18:2ω6c, 18:3ω6c) represent fungi[16,17,20], linoleic acid (18:2 ω6) is considered as reliable biomarker of fungal biomass[20]. PLFA 18:2ω6 is used biomarker for higher eukaryotes including plants[21]. PLFA 16:1ω5c represent the arbuscular mycorrhizal fungi[16,22]. Methyl branched PLFAs represent actinomycetes[14], which includes 10Me16:0, 10Me17:0, 10Me17:1ω7c, 10Me18:0, 10Me19:1ω7c and 10Me20:0[23], whereas PLFAs 14:1ω7cDMA, i15:0DMA, 16:1ω7cDMA, 18:0DMA, 18:2DMA and 19:0cy represent anaerobes[14,18,24], PLFAs 16:1ω7c and 18:1ω7c for aerobic bacteria[25], PLFAs 16:1ω7c and 16:1ω8c for methanobacter[23,26], PLFAs i17:1ω7c, 11:1ω6c, 10Me 16:0[27] and PLFA i17.0, 10Me18:0 for sulfate reducing bacteria[14,18].

Variation in PLFAs revealed the microbial community dynamics, which can be used as sensitive biomarker for periodic assessment of land degradation and progress of ecological restoration[4,28,29]. Undisturbed forest soil with defined microbial community structure in specific environmental conditions was used as reference that facilitate interpretation of the microbial community dynamics in mine spoil with increase in age of coal mine overburden. Keeping in view, the proposed study was performed to elucidate the shift in microbial community composition in coal mine spoil in due course of time in order to extrapolate the pace of mine spoil genesis following ecological restoration.

2. Materials and Methods

2.1 Study Site

Basundhara (west) open cast colliery under Ib valley of Mahanadi Coalfields Limited located in Sundargarh district of Odisha (22°03'58"-20°04'11" north latitude and 83°42'46"-83°44'45" east longitude) was selected as the study site, which was topologically hilly sloppy (244m above sea level) to plateau. The study area exhibited top soil with thickness that varies from 0.15-0.30 m with an average of 0.22 m. Climatic condition of study site is considered to be Aw according to the Köppen-Geiger climate classification, which is broadly hot, dry and semi-arid with an average of 1483 mm annual rain fall, 26°C temperature and 58.58% humidity per year. Natural vegetation of the area is considered to be tropical dry deciduous forest. However, the forest area in the site is marginally reduced due to indiscriminate coal mining activities, biotic interferences and harbor insufficient top soil to support vegetation. Coal mining activities (open cast) lead to generation of huge mine overburden spoil, which were categorized based on their age since inception namely fresh mine spoil (OB0), 3 yr (OB3), 6 yr (OB6), 9 yr (OB9), 12 yr (OB12) and 15 yr (OB15).

2.2 Mine Spoil Sampling

Each coal mine overburden was randomly demarcated into 5 blocks for the sampling of mine spoil. Pits of (15×15×15) cm^3 size were dugged up and five mine spoil samples were collected from each block from (0-15) cm soil depth that were referred as "sub-samples', which were mixed thoroughly to form one 'composite sample' for each mine overburden. Similar strategy of mine spoil sampling was performed from the chronosequence coal mine overburden (OB0 → OB15). Besides, sampling was done from nearby forest soil (NF), which was taken as reference to provide the comparative assessment. Aseptically collected samples were homogenized, sieved with 0.2 mm mesh and kept at 4°C for further analysis.

2.3 Phospholipid Fatty Acid Analysis

Lipids extraction was carried out through fractionation and quantified[30]. About 5 g dry weight mine spoil sample was sonicated using mixture of phosphate buffer, chloroform and methanol with a ratio of 4:5:10 (v/v) for 10 min with end over rotation for 2 hrs at RT. Liquid phase was transferred after centrifugation at 2500 rpm for 10 min. Equal volumes of distilled water and chloroform (5:5 v/v) was added,

mixed thoroughly and subjected to incubation for 24 hrs. After phase separation, the bottom (organic phase) was extracted under nitrogen (N_2) and kept at -20^{0}C. Solid phase chromatography was used to extract lipids by washing silica gel column with chloroform. Elution of neutral, phospholipids and glycolipids were performed using chloroform, methanol and acetone respectively through evapouration under N_2 and stored at -20^{0}C. Methanol and toluene (1:1 v/v) was used for trans-esterification of fatty acids followed by mild alkaline methanolysis using methanolic KOH for 15 min at 37^{0}C. Resulting ester-linked FAMEs were dissolved with the mixture of acetic acid, iso-octane or hexane and double distilled water having the following proportion (0.3:2:2 v/v/v) followed by vortexing for phase separation. Top (organic phase) was extracted by addition of hexane repeatedly. Combined phase was isolated under N_2 and kept at -20^{0}C which were cleaned up through SPE chromatographic technique using NH_2-SPE column. Samples were dissolved in hexane: methyl tert-butyl ether (1:1 v/v) and estimated by GC-MS.

2.4 Integrating Quotients

The ratio of gram-positive to gram-negative bacterial abundance was evaluated on the basis of PLFAs distribution in chronosequence coal mine overburden spoil and NF soil. The PLFAs i14:0, i15:0, a15:0, i16:0, 10Me16:0, i17:0, a17:0 and 10Me17:0 reflected the distribution of gram-positive bacteria whereas PLFAs 15:1ω4c, 16:1ω7c, 16:1ω9c, cy17:0, 17:1ω9c, 18:1ω7c, 18:1ω9c, cy19:0 and cy19:0ω7c for gram-negative bacteria[5,9,18,31]. Ratio of fungal to bacterial biomass (F/B) was calculated in order to determine the state of microbial communities in chronosequence coal mine spoil and NF soil. Fungal biomass was evaluated based on the distribution of PLFAs 18:1 w9c and 18:2ω6c. Total bacterial biomass was determined through the summation 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[32].

2.5 Statistical Analysis

PLFA profiling of chronosequence coal mine overburden spoil was analyzed by using Sherlock PLFA tool (Version 1.1). Shannon's diversity index or Shannon-Weaver index (H) in different mine spoil was estimated as: ($-Sp_i \ln p_i$), where p_i represents peak area of i^{th} peak. Besides, Pielou's evenness index (J) was determined as: ($H/Hmax$); where H represents the number derived from Shannon diversity index, $Hmax$ represents highest value of H ($Hmax = \ln R$; where, R represents PLFA richness). PCA was conducted using PLFAs distribution through SPSS (Version 18.0). Cluster analysis was performed based on distance matrix revealed by differential patterns of distribution of 51 PLFAs. Further, RDA was performed based on PLFAs distribution by XLSTAT-2014 (Version 2.03).

3. Results

3.1 Distribution of PLFAs

The percentage composition was estimated on the basis of 51 PLFAs distribution in chronosequence mine overburden spoil. Higher relative distribution of straight chain PLFAs was observed in OB12 (36.56%) compared to different mine spoil. The distribution of branched chain PLFAs was found to be maximum in OB0 (10.41%) and minimum in OB15 (6.19%). Microbial communities with hydroxy PLFAs exhibited decline trend from OB0 (0.36%) to OB9 (0.11%). Relative distribution of MUFAs representing aerobic prokaryotes ranged from 10.53% (OB12) to 12.31% (OB0), where as PUFAs ranged from 37.38% (OB3) to 42.83% (OB15) across the sites. The MUFAs

Table 1. Distribution of PLFAs groups (percentage composition) in chronosequence coal mine spoil (OB0 ® OB15) and NF soil

Sample	Straight	Branched	Hydroxy	MUFA	PUFA	DMA	18:1 w9c	18:2 w6c, 18:2 w9c	10-Methyl
OB0	20.23	10.41	0.36	12.31	40.51	12.65	0.65	0.28	2.63
OB3	30.08	8.35	0.28	11.46	37.38	8.69	1.23	0.54	2.31
OB6	32.12	8.15	0.15	11.21	37.84	6.67	1.67	0.91	1.84
OB9	27.36	8.18	0.11	10.64	45.12	4.58	1.75	1.15	1.32
OB12	36.56	6.31	nd	10.53	38.54	3.68	1.98	1.63	1.14
OB15	32.14	6.19	nd	11.65	42.83	2.61	3.12	1.82	0.57
NF soil	26.25	4.31	nd	10.12	51.36	1.52	4.15	2.18	0.32

nd: beyond detectable limit

and PUFAs was observed to be 10.12% and 52.36% respectively in NF soil. Decline in DMA PLFAs that ranged from 12.65% (OB0) to 2.61 (OB15) over time was also estimated. Higher dominance of fungal PLFAs 18:1w9c (4.15%) and 18:2w6, 9c (2.18%) was exhibited by NF soil compared to chronosequence coal mine overburden spoil. Methyl branched PLFAs in OB0 (2.63%) was found to be relatively higher compared to chronosequence mine overburden spoil.

3.2 Microbial Community Composition

The study revealed the shift in microbial community composition across the coal mine spoil over time and NF soil. Relative dominance of gram-positive bacteria (15.32%) and gram-negative bacteria (29.13%) was evident in OB0 in comparison to different mine spoil (Table 2). Besides, higher dominance of anaerobes in OB0 (5.12%) was due to greater relative distribution of DMA PLFAs. The relative distribution of anaerobes exhibited decline trend over time. Besides, 10.48% gram-positive, 12.43% gram-negative bacteria and 3.47% anaerobes were observed in NF soil. Methyl branched PLFAs representing actinomycetes exhibited decline trend from OB0 (2.63%) to OB15 (0.57%). The distribution of PLFA 16:1 w5c representing arbuscular mycorrhizal fungi revealed gradial increase from OB3 (0.13%) to OB15 (1.37%) over time and was found to be maximum in NF soil (4.19%) across the site. The distribution of PLFA (18:1ω9c) revealed greater fungal dominance in nearby NF soil (5.33%), which exhibited progressive improvement in fungal population from OB0

Table 2. Comparative distribution of various microbial communities (in percentage) in chronosequence coal mine spoil (OB0 * OB15) and NF soil

Sample	Gram-positive	Gram-negative	Anaerobes	ACT	A.M. Fungi	Fungi	Methano-bacter	Eukaryote
OB_0	15.32	29.13	5.12	2.63	nd	0.93	nd	47.23
OB3	13.42	21.23	4.56	2.31	0.13	1.77	nd	56.85
OB6	11.14	18.23	4.83	1.84	0.28	2.58	nd	61.19
OB9	13.81	15.85	3.85	1.32	0.39	2.91	nd	62.39
OB12	13.12	15.17	3.61	1.14	1.24	3.61	nd	62.58
OB15	11.98	14.56	3.55	0.57	1.37	4.94	nd	63.31
NF	10.48	11.43	3.47	0.32	4.19	6.33	nd	64.05

nd: beyond detectabl limit.

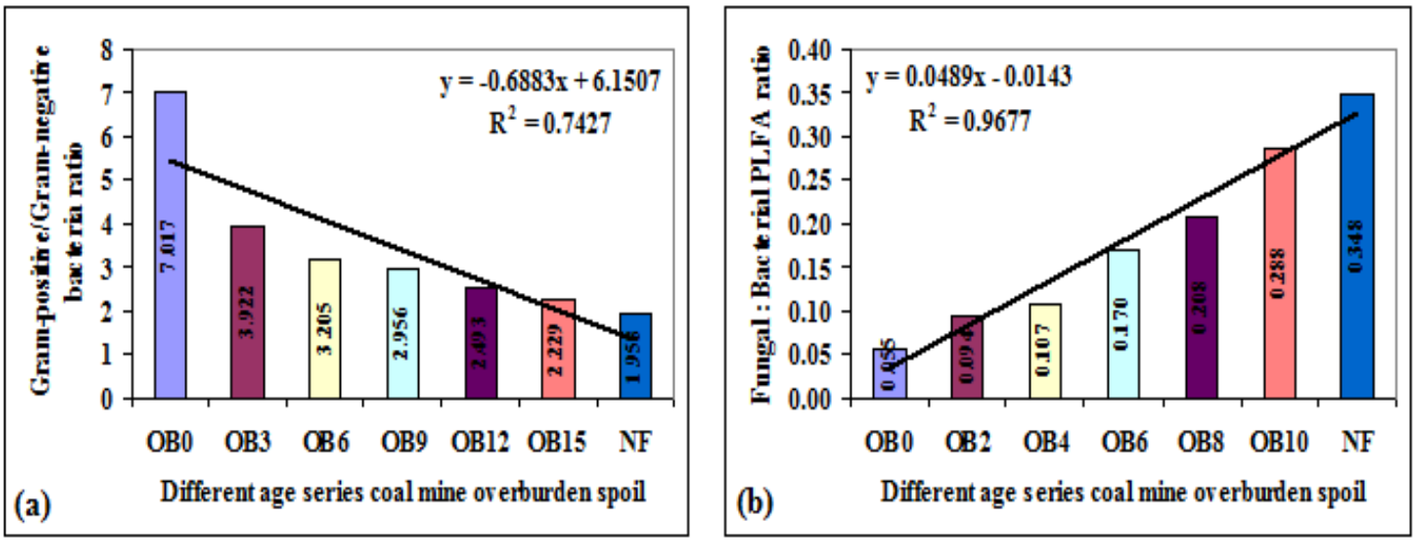

Figure 1. Integrating quotients based on the relative distribution of 51 PLFAs in chronosequence coal mine spoil (OB0 * OB15) and NF soil. (a) Ratio of Gram-positive to Gram-negative bacterial PLFAs; (b) Ratio of Fungal to Bacterial PLFAs.

(0.93%) to OB15 (4.64%). However, the relative distribution of methanobacter population in different mine spoil was beyond detectable limit. Lower longer chain fatty acids representing eukaryotic PLFAs was found to be minimum on OB0 (47.23%) whereas the maximal occurrence of relatively longer chain fatty acids revealed greater inputs from microeukaryotes in OB15 (63.31%) and was found to be maximum in the nearby NF soil (64.05%) across the sites (Table 2).

3.3 Integrating Quotients

The gram-positive to gram-negative bacterial ratio was estimated based on the distribution of PLFAs in chronosequence mine spoil that revealed gradual decline from 7.017 (OB0) to 2.229 (OB15) over time (Figure 1a). Comparatively the NF soil (1.958) exhibited lower gram-positive to gram-negative bacteria PLFAs ratio compared to chronosequence coal mine spoil. Gram-positive to gram-negative bacterial PLFAs ratio in chronosequence coal mine spoil and NF soil exhibited significant variation, which was found to be statistically significant ($r = 0.861$; $p<0.001$) (Figure 1a). Ratio of fungal to total bacterial biomass was evaluated based on the relative distribution of PLFAs in chronosequence coal mine spoil and NF soil. Further, F:B ratio showed gradual increase from OB0 (0.055) to OB15 (0.348) over time (Figure 1b). Gradual increase in F:B ratio in chronosequence coal mine overburden spoil across the sites was statistically significant ($r = 0.983$; $p<0.001$).

4. Discussion

PLFA profiling is used in detecting responses of microbial communities in various land use patterns and anthropogenic disturbances in terrestrial ecosystems[33–35]. The analysis of certain marker PLFAs representing the functional groups provide quantitative description of microbial community[18,36]. Differential patterns of response were exhibited by the existing microbial communities with differences in environmental variants, which induce variations in microbial community composition. The quantum of variation with respect to the shift in microbial community structure reflect the level of interactions between existing microbial communities and their physiological state in soil subsystem. Based on the PLFAs distribution that differ among microbial communities can be used as the potential biomarkers or fingerprints of the existing microbial community structure[12,16,17].

Microbial communities can be classified based on the relative distribution of PLFAs such as aerobic prokaryotes (MUFA), microeukaryotes (PUFA), gram-positive and anaerobic bacteria with saturated, branched fatty acids (C_{14} to C_{16}), anaerobic bacteria with saturated, branched fatty acids (C_{16} to C_{19})[19]. PUFAs are considered as biomarkers for eukaryotes where as MUFAs exhibited by gram-positive and gram-negative bacteria mostly represent aerobes. Lower level of PUFA with higher levels of MUFA[37] was reported as potential biomarkers for the gram-negative bacteria[15,38,39]. Higher occurence of gram-negative bacteria revealed the presence of hydroxyl PLFAs[15,38]. Lower level of PUFAs with dominance of unsaturated fatty acids supports bacterial dominance. Branched-chain PLFAs are considered as reliable biomarkers for anaerobes and sulfate-reducing bacteria. Higher relative dominance of *cyclopropyl* PLFAs are exhibited by the gram-negative as well as anaerobic gram-positive bacteria[39]. Besides, the presence of branched-chain *iso* and *anteiso* PLFAs are considered as the primary features of gram-positive bacteria. Moreover, the differences in MUFAs and branched PLFAs are used as suitable markers for gram-negative and gram-positive bacteria[19]. Hydroxy PLFAs present in lipid moiety of lipopolysaccharides in cell wall are considered as potential biomarkers of gram-negative bacteria[15,38]. The changes in PLFAs is due to shift in microbial community composition[29,37] in chronosequence coal mine spoil over time.

PLFAs **r**espond to anthropogenic disturbances not only alter PLFAs composition in microbial membrane (phenotypic plasticity) but also the shift in microbial community composition[12] and hence can be used as biomarkers of environmental stress. Higher level of MUFA with minimal PUFA explained the abundance of gram-negative bacteria in OB0. Dominance of gram-positive bacteria and anaerobes was estimated in OB0 due to higher existence of branched chain fatty acids[40,41]. Higher dominance of PLFAs representing gram-negative bacteria was observed in metal contaminated mine spoil[11,42,43]. Relative distribution of gram-negative, gram-positive and anaerobes exhibited decline trend in chronosequence coal mine spoil. Possible reasons of DMA PLFAs dominance in OB0 compared to chronosequence coal mine spoil is because of their ability of withstanding water stress by minimizing plasmolysis as well as accumulation of compatible solutes such as proline and glycerol for the maintenance of cell turgor[14,18,24]. Being filamentous, they can bridge air gaps between water films that exist in pore spaces during desiccation of soil[44]. Methyl-branched PLFAs represent actinomycetes[14,23], which exhibited decline trend in chronosequence coal mine spoil. Lack of 10-methyl branched PLFAs and 16:1ω8c revealed the absence of methanobacter[23] in chronosequence coal mine overburden spoil.

Dominance of arbuscular mycorrhizal fungi and fungal population was evident by higher occurrence of PLFA 16:1ω5c and PLFAs (18:1ω9c) respectively in NF soil, which is due to gradual vegetation development, plant inputs of litter and root exudates, symbiotic nitrogen fixation of localized available nutrients to support diverse microbial population[45]. Higher

existence of fungal PLFAs is supported by the availability of polymeric phenolic compounds such as lignin and tannin, and their ability towards lignin degradation and organic matter formation in NF soil[46]. PLFA 16:1ω5c exhibited by arbuscular mycorrhizal fungi substantially contribute to fungal biomass[16,17,23], which responds to changes in available C[5,47]. Lower longer chain fatty acids indicate lower input from microeukaryotes[20] in OB0 compared to chronosequence coal mine spoil. Interaction of heavy metals with membrane proteins was reported to alter protein conformation, stability and microbial activities[11,42,48].

Gradual decline in gram-negative to gram-positive PLFAs ratio may be attributed by progressive increase in gram-negative bacterial population induced by the establishment of vegetation that supplement readily available nutrients to support relatively higher microbial activities contributed by gram-negative bacteria[49]. Ration of fungal to bacterial biomass (F/B) is considered as the integrating quotient to elucidate microbial community response against ecological stresses[12]. Discrimination of the disturbed from undisturbed soil habitat based on F:B ratio was reported by several workers[8,29,44,50]. The variation in F:B ratio was less pronounced due to environmental extremities and heavy metal toxicity in chronosequence coal mine spoil[11,14,42]. Gradual increase in F:B ratio over time is attributed to higher C:N ratio supporting higher prevalence of fungal PLFAs (18:1 w9c, 18:2 w6c, 18:2w9c) in OB15. Greater F:B ratio in NF soil may be explained due to higher prevalence of fungal PLFAs and arbuscular mycorrhizal fungi (16:1 w5c) compared to chronosequence mine spoil with better adaptability to cope with available nutrients[31,50,51]. The study suggested that the disturbed ecosystems show relatively lower F:B ratio compared to organically managed soil[8]. Several studies substantiated distinct functional role of bacteria and fungi influenced by land use patterns and edaphic factors, which correlated well with microbial community composition[29,52,53].

Soil pH represents an integrating variable that can be used as sensitive predictor of microbial community composition[54]. The F:B ratio exhibited positive correlation with the variation in soil pH, which was analyzed to be statistically significant ($r = 0.984$, $p < 0.001$). Progressive increase in soil pH towards neutrality in OB15 with 96.91% variability in F:B ratio substantiated the concept[55]. Lower pH and the decline in the relative distribution of PLFAs a15:0, 16:1ω5c, 18:1ω7c, 18:1ω9c, 18:2ω6c and 18:2ω9c[55] explained the minimal F:B ratio in OB0. Besides, the changes in moisture across the sites was positively correlated with F:B ratio ($r = 0.992$, $p < 0.001$), which revealed the microbial community dynamics based on their resilience to drought tolerance[56].

Differential degree of resilience exhibited by microbes to anthropogenic disturbances contributes towards microbial community dynamics and diversity. Diversity index refers to the quantitative measure accounting existence of PLFAs richness (*R*) and their distribution patterns (evenness) that reflects the overall estimate of microbial community composition in ecology studies[43]. Greater PLFA richness was observed in OB6 (49) compared to chronosequence mine spoil (Table 3). Shannon diversity index was estimated based on PLFAs distribution representing microbial taxas that showed gradual increase from OB0 (1.52654) to OB15 (2.01391) (Table 3). NF soil (2.07463) revealed higher microbial diversity in comparison to chronosequence coal mine spoil. Microbial community composition in less disturbed ecosystem (OB15) is dynamic because of their functional responses to perturbations and resilience against shifting in microbial community composition[53]. Such variation is due to the shift in MB-C:OC ratio[57]. The evenness with respect to the distribution of microbial communities is elucidated through the estimation of Pielou's evenness index (*J*) that ranges from 0 to 1[12]. Based on the relative distribution of 51 PLFAs in chronosequence

Table 3. Shannon diversity index and Pielou's evenness index based on 51 PLFAs distribution in chronosequence coal mine spoil (OB0 ⁰ OB15) and NF soil

Site	PLFA richness (*R*)	Shannon diversity index (*H*)	Pielou's evenness index (*J*)
OB0	41	1.52654	0.41107
OB3	46	1.65823	0.43311
OB6	49	1.76237	0.45284
OB9	48	1.91393	0.49440
OB12	47	2.00056	0.51404
OB15	46	2.01391	0.52601
NF	46	2.07463	0.54187

coal mine overburden spoil, the Pielou's evenness index was evaluated that varied from 0.41107 (OB0) to 0.52601 (OB15) across the sites. The study clearly indicated that the more even with respect to the PLFAs distribution or lesser variations is observed among microbial communities in chronosequence coal mine overburden spoil, the greater is the microbial diversity. Further, the increase in diversity index solely depends on the increase in the number of types of PLFAs and evenness.

Principal component analysis was performed based on 51 PLFAs distribution in order to discriminate the chronosequence coal mine spoil and NF soil across the sites[58]. It is evident from the analysis that maximum cumulative

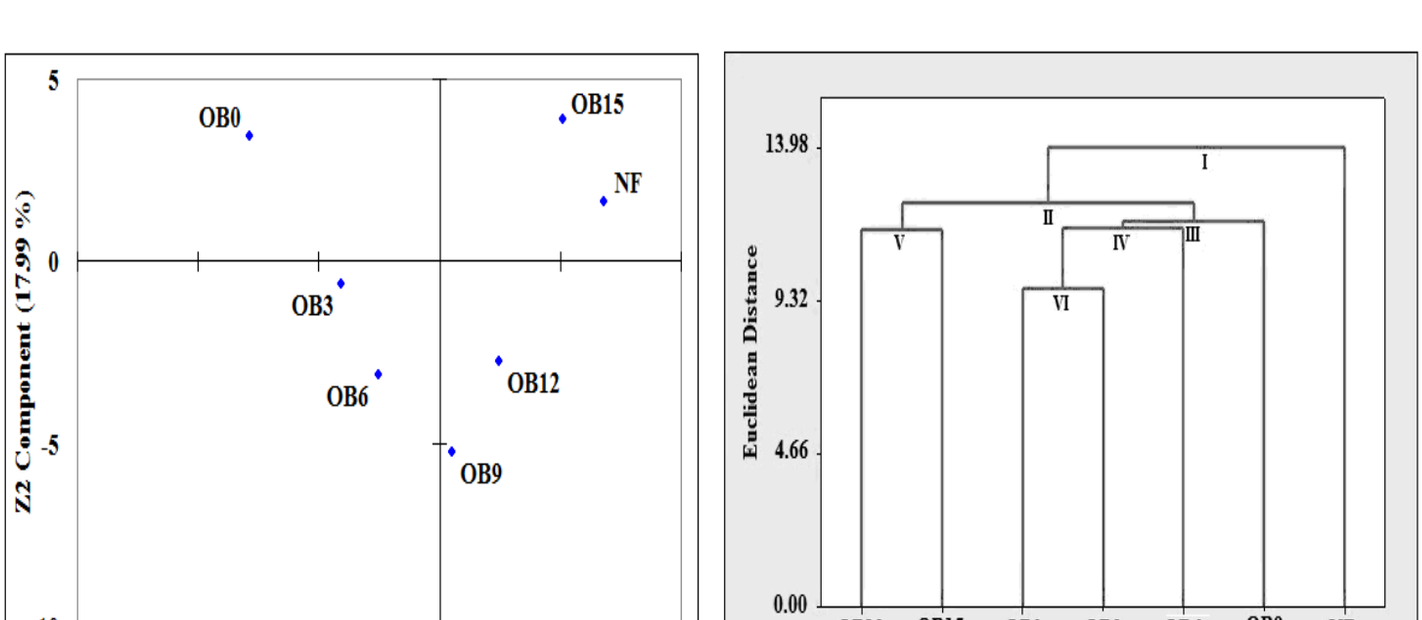

Figure 2. (a) Principal component analysis, (b) Cluster analysis based on 51 PLFAs distribution to elucidate relatedness among microbial communities in chronosequence coal mine spoil (OB0 ° OB15) and NF soil.

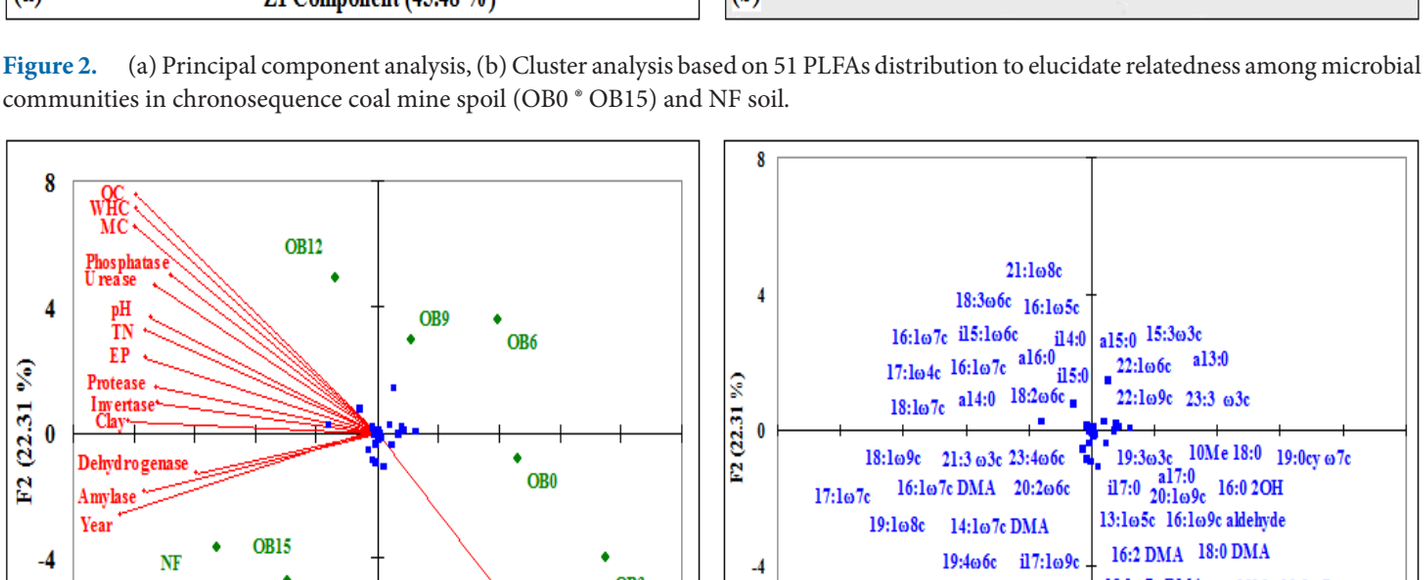

Figure 3. Redundancy analysis based on 51 PLFAs distribution and 8 microbial communities in different coal mine spoil: (a) Site codes for different coal mine spoil and NF soil; (b) PLFAs with highest scores on first two axes with additional PLFAs.

percentage of variance (63.47%) exhibited by the Z1 and Z2 components were well segregated (Figure 2a) due to the variations in microbial community structure with the increase in age of mine overburden spoil. Cluster analysis using distance matrix based on 51 PLFAs distribution in chronosequence coal mine overburden spoil over time and NF soil across the sites was performed. Existence of six independent clusters (I – VI) revealed that the likeness of unrandomized tree was well resolved statistically (Figure 2b).

RDA analysis based on PLFAs distribution explained microbial community dynamics in response to the environmental gradients including enzyme activities and physicochemical properties with the alternations in microenvironment with their efficiency to utilize the readily mineralizable resources in chronosequence mine spoil. RDA analysis explained 60.72% variability among different mine spoil profiles using the canonical sum of eigen values based on the distribution of 51 PLFAs. Environmental gradients arrows including different soil physicochemical properties and enzyme activities for RDA ordination of PLFAs was presented (Figure 3a). PLFAs with highest scores with first two ordination axes revealed significant correlation with environmental variables was displayed (Figure 3b). RDA analysis revealed that the soil attributes and enzyme activities exhibited an increasing trend towards the direction on OB12 and OB15, where as BD increased towards OB0 (Figure 3a). Certain PLFAs that correlated well with the alternations in physico-chemical properties[59] in chronosequence mine spoil was presented (Figure 3b). Prevalence of methyl-branched PLFAs such as 10Me18:0; 10Me 19:1w7c, saturated branched fatty acids including C_{16} to C_{19} and DMA PLFAs such as 16:2 DMA, 18:0 DMA, 18:1 w7c DMA revealed dominance of actinomycetes and anaerobes in OB0. Higher dominance of sulfate reducing bacteria in OB0 is due to existence of PLFA a17:0. Longer chain PLFAs representing microeukaryotes indicated minimal occurrence in OB0 due to acidic pH and metal toxicity. Higher dominance of heterotrophic microeukaryotes and arbuscular mycorrhizal fungi (16:1w5c) were estimated in OB12. Relative dominance of fungal population in OB12 and OB15 is due to higher relative distribution of PLFAs (18:2w6c, 18:3w6c) and PLFA (18:1w9c) respectively.

The present investigation indicated the microbial community dynamics in chronosequence coal mine overburden spoil profiles with gradual improvement in soil attributes in the direction of OB15 over time reflecting the progress of ecological restoration supported by mine spoil genesis. Microbial community dynamics in different age series mine overburden spoil over time may be considered as the dynamic state that responds to soil variables, microbial activities and community interactions[60] influenced by the available soil nutrients in chronosequence coal mine spoil over time. The study provides the multifaceted nature of interactions among different soil variables that influence the variations in microbial community composition in chronosequence coal mine spoil across the sites.

5. Conclusion

Due to inherent difficulties, the realistic ecological assessment necessitated the use of sensitive ecological biomarkers for monitoring the progress of ecological restoration through mine spoil genesis. The present study demonstrated that there exists microbial community dynamics among chronosequence coal mine overburden spoil over time across the sites. The heterogeneity in physicochemical attributes of coal mine spoil influence microbial community composition, which appeals an integrated approach prerequisite for monitoring mine spoil genesis. PLFA profiling is used to provide comparative study with respect to the physiological state of microbial communities, shift in microbial community structure with their efficiency to utilize readily mineralizable resources. Comparative assessment based on PLFAs distribution revealed that heavy metal toxicity resulted decline in PLFAs such as 16:1ω5c, 18:1ω7c, 18:1ω9c, 18:2ω6c, 18:2ω9c in OB_0 compared to undisturbed NF soil. RDA analysis correlates well with PLFAs distribution in response to changes in physicochemical properties in chronosequence coal mine spoil. Thus, PLFA profiling can be used as sensitive biomarkers that provide an integrative measure of microbial community structure not only useful for the assessment of ecological restoration but also implementation of appropriate soil management practices.

6. Acknowledgement

Authors were indebted to every individuals involved in sampling of mine spoil and statistical analysis during the present investigation. In the same breath, due acknowledgement has been given to the HOD, Department of Biotechnology and Bioinformatics, Sambalpur University, Odisha for providing necessary laboratory facilities and infrastructure to perform the experimental works to fulfill the objectives of the present study.

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