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# Community Level Physiological Profiling of Microbial Communities Influencing Mine Spoil Genesis in Chronosequence Coal Mine Overburden Spoil

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## ABSTRACT

Ecological restoration through mine spoil genesis should be dogmatic and the strategies involved a holistic approach, which emphasizes the role of microbial community composition that varies in accordance with the physiological and nutritional status of mine spoil profiles. This is because the patterns observed aboveground is being driven by the belowground diversity and processes. Thus, the relationship between microbial community structure and mine spoil genesis in chronosequence coal mine spoil has attracted considerable research attention. The occurrence of higher microbial diversity and difficulties in culturing microbes necessitate the use of a culture-independent approach through community-level physiological profiling based on the patterns of carbon source utilization using BIOLOG Ecoplate and thereby the functional diversity of microbial communities in different age series coal mine spoil was determined. The average well-color development exhibited an increasing trend with a minimum in OB0 (0.0640) and a maximum in OB15 (0.5060) over time. The patterns of substrate utilization (carbohydrates, carboxylic and ketonic acids, amino acids, polymers, amines, and amides) reflect the shift in microbial community composition in different age series coal mine spoil over time. Gradual increase in species richness and Shannon diversity index with the increase in age of mine spoil substantiated relatively higher microbial diversity reflecting the sign of mine spoil genesis. Principal component analysis and redundancy analysis based on the differential patterns of substrate utilization discriminate different age series coal mine spoil into independent clusters, which evaluated the broad-scale patterns of microbial community dynamics influencing the pace and progress of mine spoil genesis.

## INTRODUCTION

Mining activities result in disturbances in the landscape associated with a huge generation of mine spoil representing disequilibrated geomorphic systems, altering the microenvironment of soil microorganisms and thereby disrupting the functional stability of microbial communities (Wang et al. 2009). Ecological restoration through mine spoil genesis should be dogmatic and the strategies involved a holistic approach (Rath et al. 2010), which not only involves periodic assessment of mine spoil using microbial ecological indicators but also recovery of a degraded ecosystem with acceleration to continue as productive and sustainable ecosystem (Gasch et al. 2014). The shift in microbial communities precedes the alternations in vegetational patterns, and soil physico-chemical and microbiological properties reflect the early signs of environmental stress and ecological evolution induced by anthropogenic activities (Dangi et al. 2012). Besides, the concept of biodiversity describes spatial diversity including its link to biodiversity, and landscape ecology has emerged as the functional determinant of ecosystem processes. Ecological restoration following mine spoil genesis not only depends on mining methods, height and slope of mine overburden, nature of mine spoil, and geo-climatic conditions but also the shift in microbial community composition, resilience against ecological extremities, and microbial amelioration in coal mine spoil (Muknopadhyay et al. 2013).

Mining subsidence followed by the gradual establishment of vegetation cover exhibits a distinct influence on molecular ecological networks of microbial communities (Luo et al. 2020), which subsequently adapted to changes in distribution and activity in response to an interactive relationship with ecological processes (de Quadros et al. 2016). Being sensitive indicators of ecological restoration, mine spoil genesis focuses on the shift in microbial community composition and their relationships (Pascual et al. 2000, Harris 2003). Criteria for mine spoil genesis primarily focus on the microbial ecology of soil subsystems including microbial community dynamics, because the patterns observed aboveground are being driven by belowground diversity and processes (Kelly et al. 2003). Though the restoration of mine spoil is relatively longer, it

is a prerequisite to explore the shift in microbial community composition using sensitive soil quality biomarkers. The chronosequence study associated with the space-for-time substitutions influencing microbial community dynamics can be reliably used to assess the pace and progress of mine spoil genesis that occur between the temporally linked sites over multiple time scales (Walker et al. 2010). Microbiological properties including microbial biomass pool, enzyme activities, microbial community composition, species richness, relative distribution, and abundance of microbial communities are reliably used for monitoring mine spoil genesis (Ngugi et al. 2020).

Microbial community structure acts as an ecologically relevant endpoint and is realistically utilized for impact assessment associated with anthropogenic disturbances, which influence microbial communities at multiple spatial extents linked to environmental heterogeneity. Microbial community structure is the driving force for organic matter decomposition and nutrient turnover that regulate its size and activity (Zeller et al. 2001). The shift in microbial community composition occurs even though microbial community size remains unchanged, which reflects the role of microbial communities as a sensitive indicator of disturbances compared to measures of either microbial processes or overall community size (Veresoglou et al. 2011). Several studies explored the shift in microbial community structure along the time gradient of post-mining restoration (Li et al. 2019, Liu et al. 2019).

The approach used to detect the adaptive variations in microbial community structure in response to ecological processes caused by mining activities can be analyzed through community-level physiological profiling (CLPP). Being metabolically heterogeneous with diverse microbial metabolic pathways (Scheffer & Schachtschabel 2010), it is imperative to determine metabolic profiles for analyzing the functional diversity of microbial communities (Haferburg & Kothe 2012, Lenart-Boroń & Wolny-Koladka 2015) in chronosequence coal mine overburden spoil through community level physiological profiling using 'BIOLOG system' (Frąc et al. 2012, Rutgers et al. 2016). CLPP involves direct inoculation of environmental samples in BIOLOG$^{TM}$ eco-plate and resulting responses of NADH produced via cellular respiration that reduces tetrazolium dye to colored formazan at different time intervals of incubation provide information about functional adaptations of mixed microbial communities (Garland 1997), cell viability (Winding 1994), a measure of functional diversity (Zak et al. 1994) and overall microbial community fingerprints based on the patterns of substrate utilization (Kennedy & Gewin, 1997, Winding & Hendriksen 1997). The diversity of responses based on average well color development is a function of both richness and evenness among wells (Zak et al. 1994, Lehman et al. 1995), which determines the shift in microbial community structure and functional diversity (Frąc et al. 2012), which reveal the sign of mine spoil genesis and ecological restoration.

The forest soil with well-defined microbial communities was taken as a reference to interpret the shift in microbial community structure. The study was designed to determine the shift in microbial community structure and their functional status in chronosequence coal mine overburden spoil, which reveal the importance of metabolic diversity driving succession through microbial community dynamics that deepen the understanding of microbial interactions with mining ecosystems over time ideal for microbial ecology studies.

## MATERIALS AND METHODS

### Study Site

The present study was carried out in the Basundhara (west) open cast colliery, Ib valley area of Mahanadi Coalfields Limited (MCL) located in the revenue district of Sundargarh, Odisha, India (22°03'58"-20°04'11" north latitude and 83°42'46"-83°44'45" east longitude). Topologically, the area is hilly and sloppy (244 m above sea level) to plateau. The thickness of native top soils in the site varies from 0.15 m to 0.30 m (average: 0.22 m). The study area experiences a semi-arid climate with an annual rainfall of 1483 mm yr-1, an average temperature of 26°C, and 58.58% relative humidity. Tropical dry deciduous forest is considered to be the natural vegetation of the study site and broadly the climate is dry, hot, and arid. The open cast coal mining activities in the study site lead to the formation of different age series coal mine spoil overburdens and grouped according to their inception (Fresh mine spoil: OB0, 3 yr: OB3, 6 yr: OB6, 9 yr: OB9, 12 yr: OB12 and 15 yr: OB15).

### Mine Spoil Sampling

Each overburden was divided into 5 blocks, and from each block, five soil samples were collected from 0-15 cm depth by digging pits (15×15×15) cm$^3$ size. Samples collected from each block were referred to as 'sub-samples' and were mixed to form a 'composite sample' obtained from each overburden. A similar strategy was adapted for sampling from different age series of coal mine overburden along with forest soil (NF), which was used as a reference. Composite samples were homogenized, sieved (0.2 mm), and stored at 4°C until analyzed.

### Microbial Enumeration

Microbial enumeration of the different age series coal mine overburden spoil was performed following standard microbi-

ological approaches through serial dilution ($10^{-10}$ fold) and spread plate technique. The azotobacter count (AZB) was enumerated using azotobacter mannitol agar. The arthrobacter count (ARB) was determined using the arthrobacter medium. The rhizobial population (RZB) was estimated using yeast extract mannitol agar. The heterotrophic aerobic bacterial count (HAB) was enumerated using nutrient agar. The sulfate-reducing bacterial count (SRB) was estimated using the sulfate-reducing medium. Besides, the actinomycetes count (ACT) was determined using starch-casein agar supplemented with streptomycin and griseofulvin. The yeast population (YES) in different mine spoil samples was estimated using potato sucrose agar. The fungal count (FUN) was estimated using rose bengal agar supplemented with streptomycin.

## BIOLOG$^{TM}$ Ecoplate

Community level physiological profiling was performed using BIOLOG$^{TM}$ Ecoplate with 31 carbon sources (C1 – C31) along with control well in triplicates. Mine spoil sample (5 g) was suspended in 50 mM PBS (pH 6.5 to 7) and incubated for 1 h. After shaking, the flask was kept undisturbed for 30 mins and 150 µL of supernatant was transferred to BIOLOG$^{TM}$ Ecoplate and incubated at 45ºC. Five substrates such as (i) carbohydrates, (ii) carboxylic and ketonic acids, (iii) amino acids, (iv) polymers, and (v) amines and amides have been selected (Weber & Legge 2009). The time between sampling and inoculation should be minimized to reduce cell death. Substrate utilization rate was indicated by the reduction of tetrazolium dye that changes its color from colorless to purple was quantified (Konopka et al. 1998), if a single source was used by inoculated microbes (Lehman et al. 1995, Islam et al. 2011). Absorbance was taken at 590 nm using BIOLOG$^{TM}$ Microstation (Thermo Fischer Scientific Inc, USA) at different time intervals of incubation (24 h, 48 h, 72 h, and 96 h).

### Average Well Color Development (AWCD)

Overall color development in BIOLOG$^{TM}$ Ecoplate was expressed in terms of the average well color development (Garland & Mills 1991, Gomez et al. 2004), which is derived from the mean difference among the absorbance values of 31 response wells containing sole carbon source against control. The microbial response was expressed in terms of AWCD, which was calculated at different times of incubation (24 h, 48 h, 72 h, and 96 h) as follows.

$$AWCD = \frac{1}{31} \sum_{i=1}^{31} (A_i - A_0)$$

Where, $A_i$ represents the absorbance reading of well $i$, which is corrected by subtracting the absorbance value of blank well $A_0$ (without carbon source) from the value of each plate well.

### Species Richness and Shannon-Weaver Index

The calculated AWCD value is considered as the estimate of the total capacity of microbial communities to use sole carbon substrates, which was used to calculate different indices of microbial functional diversity (Frąc et al. 2012, Xu et al. 2015). Shortest incubation time that allows better resolution is used to calculate average well color development, richness, and Shannon-Weaver index (Frąc et al. 2012). Species richness (R) was calculated as the number of oxidized C substrates using the absorbance value of 0.25 as the threshold for a positive response (Garland 1997). Shannon-Weaver index (H) is related to the number of carbon substrates degraded by microbial communities and was calculated as H $= -\sum pi$ (ln $pi$); where, $pi$ represents the ratio of activity on each substrate to the sum of activities including all substrates.

### Data Normalization

Before performing multivariate analysis, the data was initially normalized to reduce any biasness due to differences in inoculum densities between samples (Garland 1996). Normalization of the data involves the correction of each absorbance value by its corresponding blank and then dividing by the AWCD value calculated for that time point. The normalized absorbance for a particular well ($k$) was calculated as follows.

$$\bar{A}_k = \frac{A_k - A_o}{\frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)}$$

Where, $A_i$ represents the absorbance reading of the well $i$ and $A_0$ is the absorbance reading of the blank well (without carbon source). Normalized absorbance value is coded as zero, where there is very little response or negative value in a well since this is physically meaningless.

### Statistical Analysis

Principal component analysis was performed to analyze CLPPs based on normalized absorbance data (Weber et al. 2007, Urbanová et al. 2011) using SPSS (Version 18.0). Cluster analysis was performed using the similarity matrix based on substrate utilization by microbial communities in chronosequence coal mine spoil. RDA analysis was performed to elucidate the relationship between soil variables influencing microbial community structure in chronosequence coal mine spoil using Microsoft Excel XLSTAT-2014 (Version 2.03).

## RESULTS

### Microbial Enumeration

Relative distribution of microbial populations (expressed in $\log_{10}$ CFU per g spoil) in chronosequence coal mine overburden spoil and nearby NF soil was presented (Fig. 1a-h), which revealed wide variation over time. Progressive increase in CFU in chronosequence coal mine overburden spoil with the minimum in OB0 and maximum in OB15 was exhibited by azotobacter (r = 0.980; $p<0.001$), arthrobacter (r = 0.983; $p<0.001$), rhizobia (r = 0.973; $p<0.001$),

heterotrophic aerobic bacteria (r = 0.954; $p<0.001$), actinomycetes (r = 0.953; $p<0.001$), yeast (r = 0.988; $p<0.001$) and fungi (r = 0.963; $p<0.001$), which was found to be statistically significant. In contrast, the relative distribution of sulfur-reducing bacteria exhibited a declining trend with the increase in age of coal mine overburden spoil, which was statistically significant (r = 0.973; $p<0.001$). However, the abundance and distribution of microbial populations in the nearby NF soil were found to be comparatively higher compared to different age series coal mine overburden spoil across the sites (Fig. 1a-h).
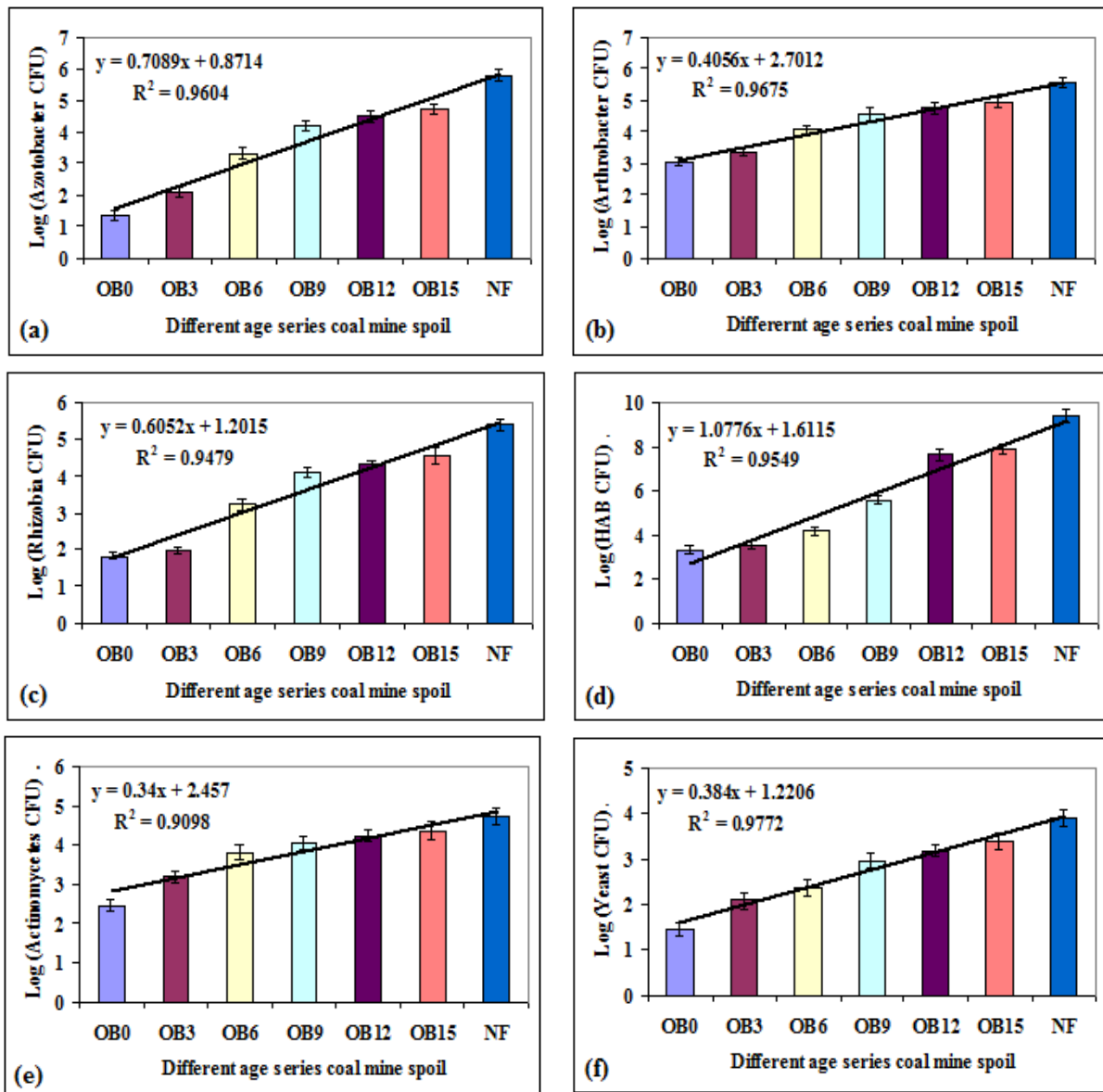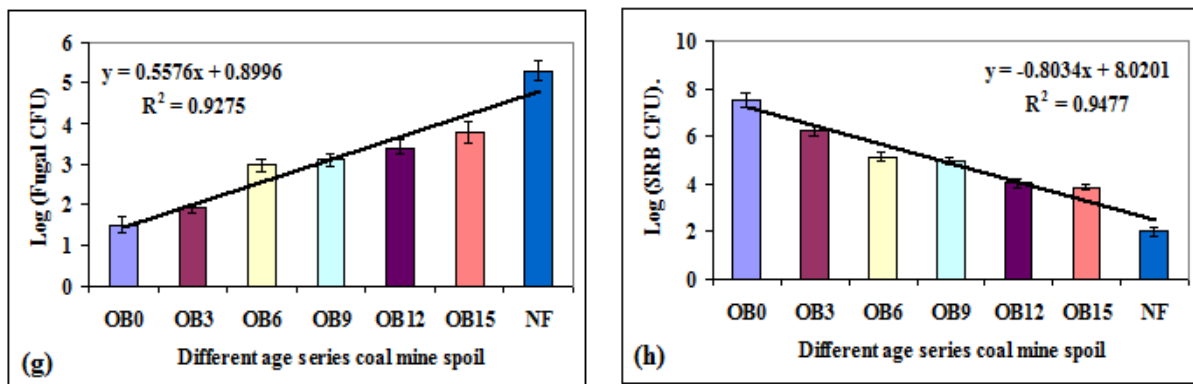
Fig. 1 (a-h): Relative distribution of microbial populations (expressed in $\log_{10}$ CFU per g spoil) in chronosequence coal mine overburden spoil over time and nearby NF soil: (a) Azotobacter, (b) Arthrobacter, (c) Rhizobia, (d) Heterotrophic aerobic bacteria, (e) Actinomycetes, (f) Yeast, (g) Fungal counts (h) Sulfate-reducing bacteria.

## Average Well Color Development (AWCD)

The average well-color development in BIOLOG$^{TM}$ Ecoplate revealed a differential pattern of responses exhibited by the microbial communities with respect to their metabolic potential in chronosequence coal mine overburden spoil, which exhibited an increasing trend with incubation time (Fig. 2). NF soil exhibited a relatively higher AWCD value compared to different age series coal mine overburden spoil over time across the sites. It is evident from the study that the shortest incubation time period (48 h) allows better resolution with respect to substrate utilization by microbial communities in chronosequence coal mine overburden spoil, which was used to calculate AWCD, species richness, and Shannon Weaver index.

## Patterns of Substrate Utilization

Community level physiological profiling of chronosequence coal mine overburden spoil was performed using BIOLOG$^{TM}$ Ecoplate, which revealed wide variation in substrate utilization such as carbohydrates (0.0033-0.4758), carboxylic and ketonic acids (0.0026-0.3267), amino acids (0.0236-0.4126), polymers (0.0004-0.2316), amines and amides (0.0077-0.0348) by microbial communities with a minimum in OB0 and maximum in OB15 over time (Fig. 3). NF soil exhibited relatively higher utilization of carbohydrates (0.6131), carboxylic and ketonic acid (0.5228), amino acids (0.5826), polymers (0.2764), amines and amides (0.0536) compared to different age series mine spoil. The physiological responses exhibited by the microbial communities in chronosequence
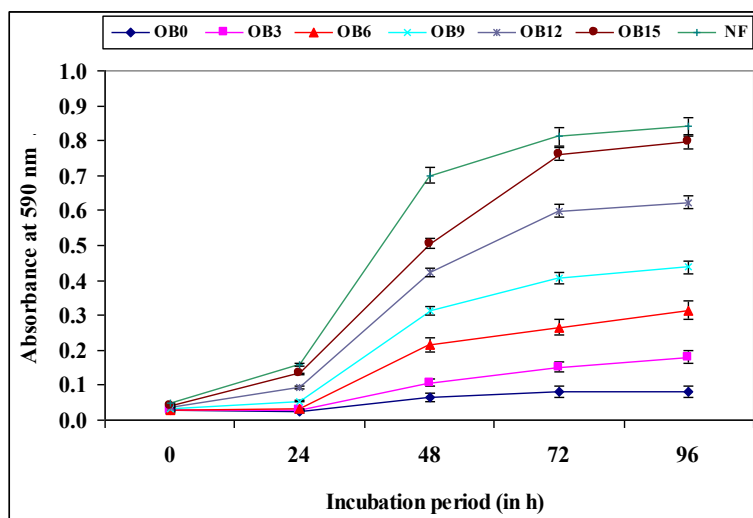


Fig. 2: Average well-color development of metabolized substrates using BIOLOG$^{TM}$ Ecoplate at different times of incubation (0, 24, 48, 72, and 96 h). Values were expressed as mean ± SD; (n =3).

coal mine overburden spoil revealed the following patterns of substrate utilization as carbohydrates > amino acids > carboxylic and ketonic acids > polymers > amines and amides over time (Fig. 3).

## Species Richness and Shannon Weaver Index

The study indicated a gradual increase in species richness from OB0 (03) to OB15 (24), which revealed relatively higher microbial diversity in OB15 with the increase in age of the mine overburden spoil across the sites (Table 1). The Shannon diversity index exhibited an increasing trend with a minimum in OB0 (0.56842) and a maximum in OB15 (2.13918) over time (Table 1). NF soil (2.27464) exhibited relatively higher microbial diversity compared to different age series mine overburden spoil. Pielou's evenness index based on the substrate utilization varied from 0.51740 (OB0) to 0.67311 (OB15) in chronosequence coal mine overburden spoil over time across the sites (Table 1).

## DISCUSSION

Wide variation in microbial community composition in chronosequence coal mine overburden spoil may be due to variation in microclimatic conditions, available soil nutrients, and heterogeneity in vegetational patterns. Being obligately aerobic and chemolithotrophic di-hydrogen fixing bacteria, the distribution of azotobacter exhibited an increasing trend from the nutrient-deficient OB0 to OB15 over time. The variability in the *Arthrobacter* population is due to nutritional versatility and starvation. The relatively higher rhizobial count was exhibited by OB15 compared to different mine

spoil due to the gradual establishment of vegetation with a symbiotic relationship with leguminous plants for nitrogen fixation, which revealed the sign of ecological restoration. Gradual decline in heterotrophic aerobic bacterial population from OB15 to OB0 is due to environmental extremities caused by extensive mining activities and heavy metal toxicity. The distribution of actinomycetes is influenced by temperature, pH, aeration, moisture, and organic C level, which may be the reason for relatively higher dominance in OB15 compared to different mine spoil. Being acid-tolerant, the minimal occurrence of actinomycetes was observed in OB0. The higher dominance of yeast in OB15 may be attributed to a gradual increase in different hydrological regimes, pH, organic C, aeration, and substrate availability over time. Being opportunistic (zymogenous), the fungal population

Table 1: Species richness, Shannon Weaver index, and Pielou's evenness index based on substrate utilization patterns by existing microbial communities in chronosequence coal mine overburden spoil over time and nearby NF soil across the sites.

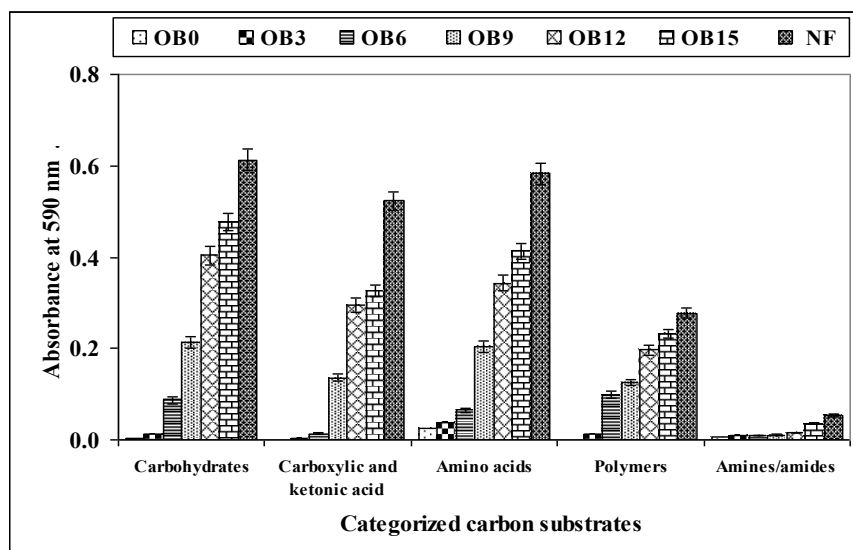| Soil profiles | Species richness (R) | Shannon Weaver index (H) | Pielou's evenness index (J) |
|---|---|---|---|
| OB0 | 3 | 0.56842 | 0.51740 |
| OB3 | 9 | 1.32470 | 0.60290 |
| OB6 | 11 | 1.51897 | 0.63346 |
| OB9 | 16 | 1.76324 | 0.63595 |
| OB12 | 22 | 1.98635 | 0.64262 |
| OB15 | 24 | 2.13918 | 0.67311 |
| NF soil | 28 | 2.27464 | 0.68262 |



Fig. 3: Differential degree of substrate utilization (C1 – C31) revealed the variation in microbial community composition in chronosequence coal mine spoil and NF soil. Values were expressed as mean ± SD; (n =3).

exists either as a free-living or mycorrhizal association to promote soil structural stability by forming macro aggregates. Relatively higher fungal dominance in OB15 may be due to the prevailing favorable moisture, improvement in pH, and available soil nutrients that enhance microbial colonization. The higher dominance of sulfur-reducing bacteria in OB0 may be due to their involvement in the utilization of sulfur compounds through oxidation of inorganic P and sulfur reduction that accounted for organic C mineralization.

Progressive improvement in AWCD from OB0 to OB15 may be due to the establishment of vegetation, available soil nutrients, and the presence of complex organic materials such as plant and root exudates (Fr c et al. 2012), which promote microbial amelioration over time. A minimal AWCD value was recorded in OB0 due to the nutrient-deficient situation with an altered geomorphic system. The chemolithotrophs mostly dominate in OB0 with heavy metal contaminants and extreme environmental conditions, which proliferate through pyrite oxidation (Jha & Singh 1991). However, the root exudates were reported to stimulate microbial proliferation associated with increased microbial activities that influence the shift in microbial community structure (Van Der Heyde et al. 2020). Relatively lower AWCD value exhibited by the chronosequence coal mine overburden spoil compared to NF soil may be due to the heavy metal toxicity that influences the relative distribution and abundance of microbial community structure (Gremion et al. 2004). A similar pattern of time adaptation toward ecosystem recovery influencing AWCD has been substantiated (Kenarova et al. 2014, Fazekaš et al. 2019, Martínez et al. 2021). Besides, the lower AWCD is due to heavy metal pollutants that restrict the availability of carbon sources for their utilization as well as toxic effects induced by metals and metalloids on microbial-mediated metabolic activities (Martínez-Toledo et al. 2021). The undisturbed NF soil harbors varieties of plant species with available soil nutrients are the possible reason for exhibiting relatively higher AWCD (Van Der Heyde et al. 2020). Microbial community composition and their activities vary in different mine spoil profiles in accordance with the physiological and nutritional status influenced by available soil nutrients and succession (Potthoff et al. 2006). The decline in microbial metabolic activities is due to the loss of microbial viability with specific functional attributes, which could be compensated by other microbial communities through microbial amelioration over time (Stefanowicz et al. 2008). Besides, the long-term adaptation of microbial growth in vitro is established using BIOLOG$^{TM}$ Ecoplate compared to the landscape-based environmental extremities revealing the inherent properties of resilience for adaptation exhibited by microbial communities (Kenarova et al. 2014, Martínez-Toledo et al. 2021). Changes in AWCD in different age series coal mine spoil revealed

improvement in the direction of OB15 through the shift in microbial community composition reflecting the progress of the ecological restoration.

Physiological responses exhibited by microbial communities revealed the following pattern of substrate utilization as carbohydrates > amino acids > carboxylic and ketonic acid > polymers > amines and amides (Kuźniar et al. 2018). Minimal substrate utilization by the microbial communities in OB0 is due to heavy metal contaminants caused by mining activities and their interference in assessing substrate for microbial-mediated activities (Tischer et al. 2008). Minimal utilization of carbohydrates in OB0 is due to the existence of chemolithotrophs as initial colonizers with a minimal tendency for carbohydrate catabolism. Colonization of other microbial populations over time may be the reason for the utilization of carbohydrates, carboxylic and ketonic acids, and amino acids as preferable nutrient sources. Microbial communities subsequently acquired the capacity to metabolize the complex and more recalcitrant substrates such as polymers, amines, and amides (Thouin et al. 2019, Chavan & Nadanathangam 2020). Several studies suggested that the catabolism of carbohydrates, carboxylic and ketonic acids and amino acids was sensitive to heavy metal toxicity (Lalor et al. 2007, Muniz et al. 2014). The study provides an insight into the multifaceted nature of physiological responses exhibited by the existing microbial community composition, which depends on available soil nutrients, the bioavailability of substrate for utilization, post-mining stress induced by heavy metal toxicity, and environmental heterogeneity that shapes the microbial community structure along with the supportive networks in chronosequence coal mine overburden spoil over time that influence the pace and progress of the ecological restoration.

The degrees of resilience exhibited by microbial communities against anthropogenic disturbances contribute to variation in microbial community composition. The diversity index is a quantitative measure used in microbial ecology studies that account for the existence of species richness (R) and distribution (evenness) as the measure of microbial community composition based on their substrate utilization using CLPP (Frac et al. 2012, Xu et al. 2015). Gradual increase in species richness in chronosequence coal mine overburden spoil revealed higher diversity among the microbial communities. Shannon diversity index exhibited an increasing trend in chronosequence coal mine overburden spoil over time, which revealed that the microbial communities in the less disturbed ecosystem may be dynamic in terms of functional responses to perturbations, but more resistant to the shift in microbial community composition (Waldrop et al. 2000). The evenness in the distribution of microbial communities is represented by Pielou's evenness index (J), which is con-

strained between 0 and 1 (Kaur et al. 2005). The increase in Pielou's evenness index in chronosequence coal mine overburden spoil suggested that the more even the distribution or less variation between microbial communities, the greater the diversity. Thus, the value of the diversity index increases when both the species richness and evenness increase.

Principal component analysis was performed based on the variation in AWCD, substrate utilization, species richness, and Shannon Weaver index of microbial community structure to discriminate the chronosequence coal mine overburden spoil and nearby NF soil across the sites (Ludwig et al. 1988). The Z1 and Z2 components explained 98.35% cumulative percentage of variance and revealed clear segregation of the chronosequence coal mine overburden spoil (Fig. 4a). Cluster analysis was performed based on ACWD using a similarity matrix, which revealed the existence of six clusters (I – VI) in the dendrogram, which exhibited the tree likeness of the original (unrandomized) tree was statistically well resolved (Fig. 4b).

Redundancy analysis based on the variations in AWCD, substrate utilization, species richness, and Shannon Weaver index explained the shift in microbial community composition based on substrate utilization patterns and AWCD in mine spoil profiles altering the microenvironment with possible effects on the efficiency of the readily mineralizable carbon substrate. About 99.05% variability in the patterns of substrate utilization was explained among microbial communities in chronosequence coal mine spoil based on fitted CLPP data from the canonical sum of eigenvalues (Fig. 5).

Site codes of mine overburden with a differential degree of substrate utilization (C1-C31) along with gradients arrows

were represented (Fig. 5). The species richness, Shannon diversity index with different microbial populations (AZB, ARB, RZB, HAB, ACT, YES) increased in the direction of OB12 and OB15, whereas the F: B ratio, FUN, and AWCD increased towards NF soil. Differential degree of different substrate utilization (C1 - C31) revealed by community level physiological profiling of chronosequence coal mine spoil suggested the shift in microbial community structure that influence ecological restoration.

## CONCLUSIONS

Microbial ecology studies provide valuable information about the shift in microbial community composition, which response quickly to natural perturbations and environmental stress due to their short generation time and intimate relationships with the biotic and abiotic components of terrestrial ecosystems attributed to their higher surface-to-volume ratio. Such studies emphasized the exposure of microbial communities to environmental extremities mediated by coal mining activities, which shift the microbial community composition with adaptive mechanisms of tolerance and substrate utilization in chronosequence coal mine overburden spoil. Although the microbial community dynamics are assessed through different ecological indices, the microbial community structure influencing ecological restoration is yet to be understood. The functional diversity of microbial communities is sensitive to environmental changes. Hence, exploration of microbial community dynamics by catabolic profiling based on substrate utilization was assessed through community-level physiological profiling, an effective approach essential for determining functional diversity influencing the pace and progress of mine spoil genesis
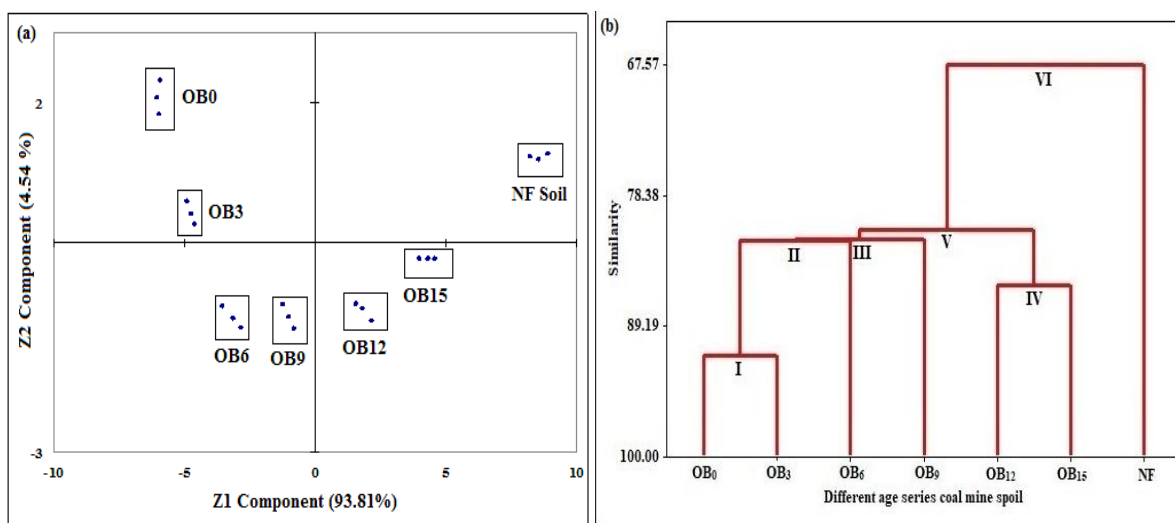


Fig. 4: (a) Principal component analysis, (b) Cluster analysis based on the variation in AWCD, substrate utilization in chronosequence coal mine overburden spoil, and NF soil.
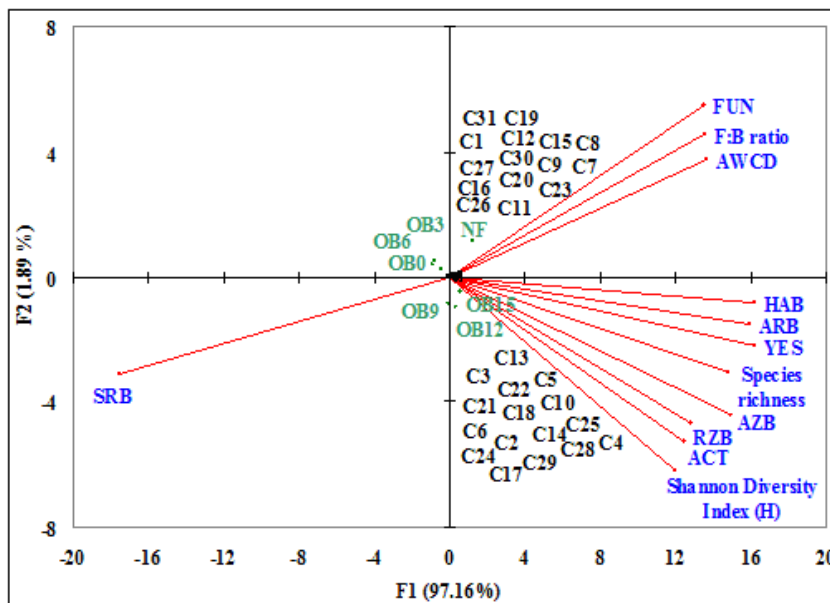
Fig. 5: Redundancy discrimination analysis based on the variation in AWCD, substrate utilization (C1 – C31), species richness, Shannon Weaver index, and different microbial CFUs in chronosequence coal mine overburden spoil and NF soil.

to support ecological restoration in chronosequence coal mine overburden spoil over time.

## ACKNOWLEDGEMENTS

## REFERENCES

Chavan, S. and Nadanathangam, V. 2020. Shifts in metabolic patterns of soil bacterial communities on exposure to metal engineered nanomaterials. Ecotoxicol. Environ. Saf., 189: 110012.

Dangi, S.R., Stahl, P.D., Wick, A.F., Ingram, L.J. and Buyer, J.S. 2012. Soil microbial community recovery in reclaimed soils on a surface coal mine site. Soil Sci. Soc. Am. J., 61: 915-924.

de Quadros, P.D., Zhalnina, K., Davis-Richardson, A.G., Drew, J.C., Menezes, F.B., Flávio, A.D. and Triplett, E.W. 2016. Coal mining practices reduce the microbial biomass, richness, and diversity of soil. Appl. Soil Ecol., 98: 195-203.

Fazekaš, J., Fazekašová, D., Adamišin, P., Huličová, P. and Benkova, E. 2019. Functional diversity of microorganisms in metal-and alkali-contaminated soils of Central and North-Eastern Slovakia. Soil Water Res., 14(1): 32-39.

Frąc, M., Oszust, K. and Lipiec, J. 2012. Community level physiological profiles (CLPP), characterization, and microbial activity of soil amended with dairy sewage sludge. Sensors, 3: 3253-3268.

Garland, J.L. 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biol. Biochem., 28(2): 213-221.

Garland, J.L. 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology. FEMS microbial. Ecol., 24(4): 289-300.

Garland, J.L. and Mills, A.L. 1991. Classification and characterization of heterotrophic microbial communities based on patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol., 57(8): 2351-2359.

Gasch, C., Huzurbazar, S. and Stahl, P. 2014. Measuring Soil Disturbance Effects And Assessing Soil Restoration Success By Examining Distributions Of Soil Properties. Appl. Soil Ecol., 76: 102-111.

Gomez, E., Garland, J. and Conti, M. 2004. Reproducibility in the response of soil bacterial community-level physiological profiles from a land use intensification gradient. Appl. Soil Ecol., 26(1): 21-30.

Gremion, F., Chatzinotas, A., Kaufmann, K., Von Sigler, W. and Harms, H. 2004. Impacts of heavy metal contamination and phytoremediation on a microbial community during a twelve-month microcosm experiment. FEMS Microbiol. Ecol., 48(2): 273-283.

Haferburg, G. and Kothe, E. 2012. Biogeosciences in Heavy Metal-Contaminated Soils. In Kothe, E. and Varma, A. (eds), Bio-Geo Interactions in Metal-Contaminated Soils. Springer, The Netherlands, pp. 17-34.

Harris, J.A. 2003. Measurements of the soil microbial community for estimating the success of the restoration. Eur. J. Soil Sci., 54(4): 801-808.

Islam, R., Chauhan, P., Kim, Y., Kim, M. and Sa, T. 2011. Community level functional diversity and enzyme activities in paddy soils under different long-term fertilizer management practices. Biol. Fertil. Soils., 47(5): 599-604.

Jha, A.K. and Singh, J.S. 1991. Spoil characteristics and vegetation development of an age series of mine spoil in a dry tropical environment. Vegetation, 97(1): 63-76.

Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R. and Kaushik, R. 2005. Phospholipid fatty acid–a bioindicator of environment monitoring and assessment in the soil ecosystem. Curr. Sci., 11: 1103-1112.

Kelly, J.J., Häggblom, M.M. and Tate, R.L. 2003. Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter as indicated by analysis of microbial community phospholipid fatty acid profiles. Biol. Fertil. Soils., 38(2): 65-71.

Kenarova, A., Radeva, G., Traykov, I. and Boteva, S. 2014. Community level physiological profiles of bacterial communities inhabiting uranium mining-impacted sites. Ecotoxicol. Environ. Saf., 100: 226-232.

Kennedy, A.C. and Gewin, V.L. 1997. Soil microbial diversity: Present and future considerations. Soil Sci., 162(9): 607-617.

Konopka, A., Oliver, L. and Turco, Jr. R.F. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. Microb. Ecol., 35(2): 103-115.

Kuźniar, A., Banach, A., Stępniewska, Z., Frąc, M., Oszust, K., Gryta, A., Kłos, M. and Wolińska, A. 2018. Community-level physiological profiles of microorganisms inhabiting soil contaminated with heavy metals. Int. Agrop., 32(1): 101-109.

Lalor, B.M., Cookson, W.R. and Murphy, D.V. 2007. Comparison of two methods that assess soil community level physiological profiles in a forest ecosystem. Soil Biol. Biochem., 39(2): 454-462.

Lehman, R.M., Colwell, F.S., Ringelberg, D.B. and White, D.C. 1995. Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. J. Microbiol. Methods., 22(3): 263-281.

Lenart-Boroń, A. and Wolny-Koładka, K. 2015. Heavy metal concentration and the occurrence of selected microorganisms in soils of a steelworks area in Poland. Plant Soil Environ., 61(6): 273-278.

Li, P., Zhang, X., Hao, M., Cui, Y., Zhu, S. and Zhang, Y. 2019. Effects of vegetation restoration on soil bacterial communities, enzyme activities, and nutrients of reconstructed soil in a mining area on the Loess Plateau, China. Sustainability, (8): 2295-2311.

Liu, Y., Lei, S. and Gong, C. 2019. Comparison of plant and microbial communities between an artificial restoration and natural restoration topsoil in coal mining subsidence area. Environ. Earth Sci., 78(6): 1-3.

Ludwig, J.A., Reynolds, J.F., Quartet, L. and Reynolds, J.F. 1988. Statistical Ecology: A Primer on Methods and Computing. Wiley-Interscience Pub., New York, pp. 331-337.

Luo, R., Kuzyakov, Y., Liu, D., Fan, J., Luo, J., Lindsey, S., He, J.S. and Ding, W. 2020. Nutrient addition reduces carbon sequestration in Tibetan grassland soil: disentangling microbial and physical controls. Soil Biol. Biochem., 144: 107764.

Martínez-Toledo, Á., González-Mille, D.J., García-Arreola, M.E., Cruz-Santiago, O., Trejo-Acevedo, A. and Ilizaliturri-Hernández, C.A. 2021. Patterns in the utilization of carbon sources in soil microbial communities contaminated with mine solid wastes from San Luis Potosi, Mexico. Ecotoxicol. Environ. Saf., 208: 111493.

Mukhopadhyay, S., Maiti, S.K. and Masto, R.E. 2013. Use of reclaimed mine soil index (RMSI) for screening of tree species for reclamation of coal mine degraded land. Ecol. Eng., 57: 133-142.

Muniz, S., Lacarta, J., Pata, M.P., Jimenez, J.J. and Navarro, E. 2014. Analysis of the diversity of substrate utilization of soil bacteria exposed to Cd and earthworm activity using generalized additive models. PLoS One, 9(1): e85057.

Ngugi, M.R., Fechner, N., Neldner, V.J. and Dennis, P.G. 2020. Successional dynamics of soil fungal diversity along a restoration chronosequence post-coal mining. Restor. Ecol., 28(3): 543-552.

Pascual, J.A., Garcia, C., Hernandez, T., Moreno, J.L. and Ros, M. 2000. Soil microbial activity as a biomarker of degradation and remediation processes. Soil Bio. Biochem., 32(13): 1877-1883.

Potthoff, M., Steenwerth, K.L., Jackson, L.E., Drenovsky, R.E., Scow, K.M. and Joergensen, R.G. 2006. Soil microbial community composition is affected by restoration practices in California grassland. Soil Biol. Biochem., 38(7): 1851-1860.

Rath, M., Mishra, C.S. and Mohanty, R.C. 2010. Microbial population and some soil enzyme activities in iron and chromite mine spoil. Int. J. Ecol. Environ. Sci., 36(2/3): 187-193.

Rutgers, M., Wouterse, M., Drost, S.M., Breure, A.M., Mulder, C., Stone, D., Creamer, R.E., Winding, A. and Bloem, J. 2016. Monitoring soil bacteria with community-level physiological profiles using Biolog™ ECO-Plates in the Netherlands and Europe. Appl. Soil Ecol., 97: 23-35.

Scheffer, F. and Schachtschabel, P. 2010. Bodenmikroflora. Lehrbuchder Bodenkunde (in German) (Eds F. Scheffer, P. Schachtschabel). Spektrum Akademischer Verlag, Heidelberg. pp. 84-99.

Stefanowicz, A.M., Niklińska, M. and Laskowski, R. 2008. Metals affect soil bacterial and fungal functional diversity differently. Environ. Toxicol. Chem. Int. J., 3: 591-598.

Thouin, H., Norini, M.P., Le Forestier, L., Gautret, P., Motelica-Heino, M., Breeze, D., Gassaud, C. and Battaglia-Brunet, F. 2019. Microcosm-scale biogeochemical stabilization of Pb, As Ba, and Zn in mine tailings amended with manure and ochre. Appl. Geochem., 111: 104438.

Tischer, S., Tanneberg, H. and Guggenberger, G. 2008. Microbial parameters of soils contaminated with heavy metals: Assessment for ecotoxicological monitoring. Pol. J. Ecol., 56(3): 471-479.

Urbanová, M., Kopecký, J., Valášková, V., Ságová-Marečková, M., Elhottová, D., Kyselková, M., Moënne-Loccoz, Y. and Baldrian, P. 2011. Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining. FEMS Microbiol. Ecol., 78(1): 59-69.

Van Der Heyde, M., Bunce, M., Dixon, K., Wardell-Johnson, G., White, N.E, and Nevill, P. 2020. Changes in soil microbial communities in post mine ecological restoration: implications for monitoring using high throughput DNA sequencing. Sci. Total Environ., 749: 142262.

Veresoglou, S.D., Mamolos, A.P., Thornton, B., Voulgari, O.K., Sen, R. and Veresoglou, D.S. 2011. Medium-term fertilization of grassland plant communities masks plants species-linked effects on soil microbial communities structure. Plant Soil, 344(1): 187-196.

Waldrop, M.P., Balser, T.C. and Firestone, M.K. 2000. Linking microbial community composition to function in a tropical soil. Soil Biol. Biochem., 32(13): 1837-1846.

Walker, L.R., Wardle, D.A., Bardgett, R.D. and Clarkson, B.D. 2010. The use of chronosequences in studies of ecological succession and soil development. J. Ecol., 98(4): 725-736.

Wang, L.P., Zhang, W.W., Guo, G.X., Qian, K.M. and Huang, X.P. 2009. Selection experiments for the optimum combination of AMF-plant-substrate for the restoration of coal mines. Mining Science and Technology (China), 19(4): 479-482.

Weber, K.P. and Legge, R.L. 2009. The one-dimensional metric for tracking bacterial community divergence using sole carbon source utilization patterns. J. Microbiol. Methods., 79(1): 55-61.

Weber, K.P., Grove, J.A., Gehder, M., Anderson, W.A. and Legge, R.L. 2007. Data transformations in the analysis of community-level substrate utilization data from microplates. J. Microbiol. Methods, 69(3): 461-469.

Winding, A. 1994. Fingerprinting Bacterial Soil Communities Using Biolog Microtitre Plates. In Ritz, K., Dighton, J., and Giller, K.E. (eds.), Beyond the Biomass: Compositional and Functional Analysis of Soil Microbial Communities. John Wiley & Sons, Chichester (United Kingdom), pp. 85-94.

Winding, A. and Hendriksen, N.B. 1997. Biolog Substrate Utilisation Assay For Metabolic Fingerprints Of Soil Bacteria: Incubation Effects. In Insam, H. and Rangger, A. (eds), Microbial Communities: Function Versus Structural Approaches, Springer, The Netherlands. pp. 195-205.

Xu, W., Ge, Z. and Poudel, D.R. 2015. Application and optimization of Biolog eco plates in functional diversity studies of soil microbial communities. MATEC Web Conf., 22: 04015.

Zak, J.C., Willig, M.R., Moorhead, D.L. and Wildman, H.G. 1994. Functional diversity of microbial communities: a quantitative approach. Soil Biol. Biochem., 26(9): 1101-1108.

Zeller, V., Bardgett. R.D. and Tappeiner, U. 2001. Site and management effects on soil microbial properties of subalpine meadows: A study of land abandonment along a north-south gradient in the European Alps. Soil Biol. Biochem., 33(4-5): 639-649.