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Assessment of microbial biomass pool in chronosequence coal mine spoil used as biomarker of mine spoil genesis

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ABSTRACT: Extensive coal mining activities result mine spoil generation dumped in form of overburdens altering biogeochemical cycles and land degradation. Mine spoil generated after post-mining activities associated with heavy metal toxicity inhibit microbial growth. Being deficient in available nutrients due to lack of biologically rich topsoil, mine spoil represents a disequilibrated geomorphic system and poses problems for revegetation and restoration of coal mine spoil. Mine spoil genesis influencing ecosystem functionality demands physicochemical characterization and spatial distribution of microbial biomass pool in chronosequence coal mine spoil. Progressive improvement in clay, hydrological regimes, OC, TN and EP microbial biomass pool and BSR over time was evident from the study. Time dependent increase in integrating quotients was used to monitor the progress of mine spoil genesis. Decline in microbial metabolic quotient over time revealed the progress of mine spoil genesis. Stepwise multiple regression analysis revealed the contribution of physicochemical attributes influencing variability in microbial biomass. The shift in physicochemical properties and microbial biomass correlated well with the extent of land degradation, which can be used as effective biomarkers for monitoring the pace and progress of mine spoil genesis. The fresh coal mine spoil to attain soil features of nearby forest soil through mine spoil genesis shall take approximately 29.15 years. The study paves the way of greater understanding not only in the direction to design appropriate management strategies for ecosystem restoration but also to improve soil quality for sustainable development.

Keywords: Mine Spoil, Physicochemical Properties, Microbial Biomass, Mine Spoil Genesis.

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1. INTRODUCTION

Being the reservoir of soil nutrients, moisture and energy sources, soil represents a dynamic and heterogeneous system that determines vegetation development supported by microbial colonization, mineralization and decomposition of organic matter leading to the creation of suitable habitat for subsequent colonization of microbial communities over time. Continuous interactions between soil minerals, organic matter and microorganisms influence different physicochemical and biological properties of the terrestrial ecosystem, and hence are considered as the critical controlling component for ecosystem functioning. However, the extensive open cast coal mining activities result in drastic alterations in biogeochemical cycles leading to land degradation with adverse changes in textural composition, structural stability and its biological attributes through the generation of huge mine spoil dumped in the form of overburdens. Mine spoil generated after post-mining activities refer to the mixture of coal seam, parent rock and subsoil with heavy metal contaminants.[1] Being deficient in available nutrients due to the lack of biologically rich topsoil, the mine overburden spoil represents disequilibrated geomorphic system[1] and poses problems for pedogenesis, revegetation and restoration of coal mine overburden spoil.[1,2,3,4] The slow recovery of mine spoils restoration due to constraints in microbial growth and proliferation and vegetational succession has been reported.[1,4,5] Ecological restoration through mine spoil genesis should be dogmatic and involves holistic approach, which not only use reliable soil quality indicators for periodic assessment over time, but also assist the recovery of degraded ecosystem with acceleration to continue as self-sustaining ecosystem.[6,7]Soil quality assessment from ecological standpoints in broader perspective encompasses the physicochemical characterization such as textural composition, hydrological regimes (bulk density, water holding capacity and moisture), soil pH, organic C, total N and extractable P, which are used as early and sensitive indicators of mine spoil genesis and dynamic changes in ecosystem recovery.[8,9,10] Coal mine overburden spoil created aftermath of mining activities represents rigorous conditions for both plant and microbes leading to the decline in soil quality and productivity in terrestrial ecosystem. Thus, the periodic assessment of mine spoil genesis using sensitive biomarkers is prerequisite in order to implement the appropriate management strategies for restoration of the legacy of mining sites into the soil features of nearby forest soil over time. Physicochemical properties define substrate availability, energy source for microbes and dynamic changes in microbial community structure necessary to sustain ecosystem functions. Clay represents the main source of available soil nutrients, which influences the physical and mechanical behaviour of soil attributes relating to different hydrological regimes (bulk density, water holding capacity, moisture) influencing functional attributes in different soil profiles.[11,12] The hydrological regimes such as bulk density, water holding capacity, moisture affect the available soil nutrients and spatial distribution of microbial community influencing the microbial mediated processes and productivity in terrestrial ecosystems.[13,14,15]Acidity of soil pH contributes long-lasting effects on textural composition, nutrients availability and microbial community structure.[15]The availability of organic C is based on the size and breakdown of SOM that triggers nutrient availability through mineralization[1]associated with mine spoil genesis while their distribution pattern influence ecosystem functions.[15,16,17] Deficiency of nitrogen as micronutrient caused by mining activities limits the establishment of vegetation,[4] and shift in microbial community structure.[18,19,20] Implication towards increasing soil quality, phosphorous is required by the plants as macronutrient that occur both in inorganic and organic forms through mineralization.[16,19]Microbial biomass is the living part of soil organic matter that constitutes a transformation matrix for organic matter and acts as labile reservoirs for plant available C, N and P.[21] Microbial biomass pool reflects the degree of immobilization of carbon and nitrogen in soil subsystems. The size of microbial biomass pool is considered as the functional index of soil quality,[16,20] ecological marker of soil health[22] and labile pool of plant available nutrients[21,23] that determine the microbial community structure to support the nutrient conserving mechanisms through immobilization and mineralization.[24]The decline in microbial biomass could result in mineralization of nutrients while the increase in pool size of microbial biomass leads to immobilization.[25]Relatively higher organic C and microbial biomass supplement greater functional diversity among microbial communities in different soil profiles.[26] Thus, the periodic assessment of microbial biomass pool is considered as valuable designator to monitor mine spoil genesis in different soil systems.Microbial biomass C comprises of 1-5% of organic C, which responds more rapidly to alteration in soil quality compared to organic matter and correlated well with decomposition of soil organic matter, nutrient cycling, structural stability.[26,27] MB-C protects soil from degradation and considered as sensitive index of the changes in organic C associated with vegetation cover and species richness.[16,28]MB-N constitutes the significant part of potentially mineralizable N and serves both as the transformation agent and sink/source of nitrogen.[23] Consequently, MB-N has the importance in quantifying nitrogen dynamics in terrestrial ecosystems as it regulates the nitrogen availability and overall nitrogen cycling.[29,30] The rapid turnover of microbial biomass P in microbial biomass pool accounted 2-10% of total P,[31] which contributes the major source to available P for vegetation development. Microbial immobilization of inorganic phosphorus in soil protects the available phosphorus from physicochemical fixation.[16,28]Microbial basal soil respiration represents the overall microbial activities reflecting the extent of decomposition and availability of slow flowing carbon through mineralization,[32] which can be used as potential index to assess microbial activities and microbial turnover rate influencing ecosystem functioning.[33] Microbial metabolic quotient or ‘biomass specific reaction activity' is determined in order to assess the degree of biological perturbations in soil systems, which is expressed in terms of CO_2-C evolved per unit of microbial biomass C per unit time.[34,35] Soil disturbances decrease microbial efficiency and increase microbial metabolic quotient as microbes require more energy to maintain the biological equilibrium and thereby predict the progress of mine spoil genesis. The restoration of coal mine overburden spoil requires periodic monitoring of the progress of mine spoil genesis, which is influenced by the physicochemical properties and microbial biomass dynamics. Keeping in view, the present study was designed to determine the progress of mine spoil genesis in six different age series coal mine overburden spoil in chronosequence (OB0 → OB15) over time as well as nearby NF soil as the reference through the comparative assessment of physicochemical properties, microbial biomass pool, basal soil respiration and certain integrating quotients (MB-C:OC, MB-N:TN, MB-P:EP, BSR:OC and microbial metabolic quotient) with an aim to elucidate not only the pace and progress of mine spoil genesis, but also pave the greater understanding in the direction of improving soil quality prerequisite for ecosystem functioning.

2. MATERIALS AND METHODS

2.1 Study site

The present study was carried out in the Basundhara (west) open cast colliery, Ib valley area of Mahanadi Coalfields Limited (MCL), Sundargarh, Odisha (22°03'58"-20°04'11" north latitude and 83°42'46"-83°44'45" east longitude). Topologically, the area is hilly and sloppy (244m 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). Climatic condition of the site is considered to be Aw according to the Köppen-Geiger climate classification. The area experiences semi-arid climate with annual rainfall of 1483 mm yr-1, average temperature of 26°C and relative humidity of 58.58%. Tropical dry deciduous forest is considered to be the natural vegetation of the study site and broadly the climate is dry, hot and arid. Because of mining activities and biotic interference, the forest area is marginally reduced and harbors insufficient organic top soil to support revegetation. Open cast mining activities 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).

2.2 Sampling of mine spoil

Each overburden was divided into 5 blocks, and from each block, five spoil 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 overburdened site. Similar strategy has been followed for sampling from six different age series of coal mine overburden (OB0 → OB15) along with the nearby native forest soil (NF), which was used as reference. Composite samples were homogenized, sieved (0.2 mm) and stored at 4°C until analyzed.

2.3 Physicochemical characterization

The clay percentage (≤ 0.002 mm) in different mine spoils and nearby NF soil was analyzed based on prescribed methodology in 'Tropical soil biology and fertility hand book'.[36,37] The hydrological regimes such as bulk density, water holding capacity and moisture content were also determined. Bulk density (BD) was calculated as [weight of excavated spoil (in g)/volume of sand (cm^3)] following the TSBF Handbook.[37] Water holding capacity (WHC) was determined[36] and expressed in percentage. Moisture content (MC) was determined by gravimetric method[36] through oven drying and expressed in percentage. The pH of mine spoil was determined using a digital pH meter (Make: Systronics, Model: MK VI).

2.4 Estimation of Organic Carbon

Organic carbon (OC) content was estimated following titration method.[36]To 5g of oven dried mine spoil, 10mL of 1N $K_2Cr_2O_7$ and 20mL of conc. H_2SO_4 were added in a 500mL Erlenmeyer flask. Soil suspension was shaken thoroughly for 5min and allowed to stand for 30 min. Suspension was diluted with 200mL of distilled water followed by addition of 1mL of 85% H_3PO_4 and 1mL of diphenylamine indicator. Mixture was then titrated against 1N $(NH_4)_2Fe(SO_4)_2.6H_2O$ until the mixture flashed to green. Thereafter, 0.5mL of 1N $K_2Cr_2O_7$ was added and titration was completed by adding 1N $(NH_4)_2Fe(SO_4)_2.6H_2O$ till last traces of blue colour disappeared. Organic C content (mg C/g spoil) was estimated as: $[(V_1\text{-}V_2)/ W] \times 0.003$; where V_1 = vol. of 1N $K_2Cr_2O_7$, V_2 = vol. of 1N $(NH_4)_2Fe(SO_4)_2.6H_2O$, W = wt. of mine spoil sample.

2.5 Estimation of Total Nitrogen

Total nitrogen (TN) was determined by Kjeldahl method.[38] About 10g of mine spoil was moistened with 25mL of distilled water for 30 min in a 300mL Kjeldahl flask followed by addition of 20g of sodium sulfate and catalyst mixture solution (20g copper sulfate, 3g of mercuric oxide, 1g selenium power). To one part, 20 parts of anhydrous sodium sulfate and a pinch of granulated zinc were added followed by 35mL of conc. H_2SO_4 and subjected to mild heat treatment for 30 min for complete digestion. After cooling, 100mL of water was added, allowed to stand for 5 min and supernatant was transferred to 500mL conical flask followed by addition of 25mL of 4% boric acid and 5 drops of mixed indicator (0.5g bromocresol green and 0.1g methyl red in 100mL of 95% ethyl alcohol). The condenser was connected to the flask and 100mL of 40% NaOH was added slowly. By heating the mixture, 150 mL of distillate was collected and titrated against N/14 H_2SO_4 till the faint pink coloration was reached. Similarly, blank was run without a spoil sample. Total N content (µg N/g spoil) was calculated: $[(T\text{-}B) \times N \times 14.007]/ W$; where T and B = volume of titrants used against sample and blank, N = normality of titrant and W = wt. of spoil sample.

2.6 Estimation of Extractable Phosphorus

The extractable phosphorus (EP) was estimated through chlorostannous reduced molybdophosphoric blue colour method in HCl.[39] To 5g of mine spoil, 50mL of 0.03N NH_4F in 0.025N HCl was added, shaken for 5 min and filtered. To 2.5mL of filtrate, 7.5mL of ammonium molybdate was added followed by 0.5mL of $SnCl_2$ and allowed to stand for (4-20) min. Absorbance was measured at 660 nm against control and the extractable P content in mine spoil sample was expressed in µg P/g spoil.

2.7 Microbial Biomass Carbon

Mine spoil samples were stored at 28 ± 2°C for a week to stabilize the respiration and subsequently used for the estimate of microbial biomass. Microbial biomass carbon (MB-C) was determined by fumigation extraction method.[40]Two portions of moist soil sample equivalent to 25g on oven dry weight basis were taken. One portion was fumigated with ethanol-free chloroform for 24 h at 25°C, extracted with 100mL of 0.5M K_2SO_4 for 30 min by horizontal shaking at 200 rpm and filtered through Whatman No. 42. The unmitigated portion was extracted at the time of fumigation. The filtered extracts were preserved at -20°C. Organic C content in the filtrate (8 mL extract) was determined by wet oxidation with 2mL of 0.4N $K_2Cr_2O_7$ in presence of 15mL of acid mixture (H_2SO_4/H_3PO_4). Mixture was transferred to 250mL of round bottom flask fitted with the Leidig condenser and gently refluxed for 30 min, cooled and diluted with 25mL of water. The residual dichromate was measured through back titration with 0.04N $(NH_4)_2Fe(SO_4)_2.6H_2O$ using a ferroin indicator. Microbial biomass carbon (MB-C) was calculated by subtracting the organic C content in extract of unmitigated sample from fumigated sample and divided by K_{EC} factor (fraction of biomass C extracted after fumigation) having the value of 0.38 (Brookes *et al.,* 1985). All the estimations were expressed on an oven dry weight basis.

2.8 Microbial Biomass Nitrogen

Microbial biomass nitrogen (MB-N) was estimated by $CHCl_3$ fumigation.[41]About 10g spoil sample was fumigated with chloroform and incubated for 24 h. Then, 50 mL of 0.5M K_2SO_4 was added to the suspension, shaken for 30min in a rotary shaker and filtered through Whatman No. 42. Similarly, the extract of unfumigated mine spoil was prepared, which was used as control. Total nitrogen content in K_2SO_4 extract of fumigated and unfumigated sample was measured after Kjeldahl digestion. Microbial biomass nitrogen (MB-N) was calculated by subtracting the total N content in K_2SO_4 extract of unfumigated sample from fumigated sample and divided by K_N factor (fraction of biomass N extracted after fumigation) having the value of 0.54 (Brookes *et al.,* 1985). All the estimations were expressed on oven dry weight basis.

2.9 Microbial Biomass Phosphorous

Microbial biomass phosphorus (MB-P) was estimated,[42] where inorganic P was extracted by 0.5M $NaHCO_3$ (pH 8.5) adjusted with NaOH. Extracted P was determined by $CHCl_3$ fumigation.[31] About 10g of sample was fumigated with $CHCl_3$ for 24 h, transferred to 250mL conical flask followed by addition of 200mL of P extractant and 2g of activated charcoal. Suspension was mixed thoroughly for 30 min and filtered. About 10mL of chloromolybdic acid reagent was added to 10mL of filtrate in a 50mL volumetric flask, gently swirled to expel out CO_2 and the volume was made upto 40mL by adding water followed by 2mL of $SnCl_2$. Similarly, the unfumigated spoil sample (control) was prepared. Absorbance was taken at 660nm preferably 10 min after the addition of $SnCl_2$ using UV-VIS spectrophotometer (Make: Systronics 117). Phosphorus content in spoil sample was calculated from the standard curve using KH_2PO_4. Microbial biomass phosphorus (MB-P) was calculated using $NaHCO_3$ extractable P from the fumigated sample minus that extracted from unfumigated sample and then divided by Kp factor (fraction of biomass P extracted after fumigation) having value of 0.40. MB-P was further corrected for P fixation during $NaHCO_3$ extraction by measuring the exogenously added P (as KH_2PO_4 equivalent to 20µg/g spoil). All the estimations were expressed on oven dry weight basis.

2.10 Basal soil respiration rate

Microbial basal respiration was estimated by alkali absorption method.[43]To 10g moist spoil sample, 1mL of 1% glucose followed by 1mL $(NH_4)_2SO_4$ were added. Thereafter, 5mL of 0.05N NaOH was added to trap the CO_2. Same procedure was followed without mine spoil (for blank). Sample and blank were incubated at 28°C for 24 h and titrated against 1N HCl with $BaCl_2$ and phenolphthalein indicator. Microbial basal respiration (µg CO_2-C g^{-1} dry soil $h^{-}1$ at 28°C) was estimated as follows: [{(Vo – V) x S x 22 x 1000 x 12}/ (M x dry wt. of sample x t x 44)]; where Vo and V = volume of HCl consumed during titration of blank and sample respectively, S = strength of HCl (normality), t = incubation time, M = wt. of sample and 22 = equivalent wt. of CO_2.

2.11 STATISTICAL ANALYSIS

The composite mine spoil samples collected from six different age series coal mine overburden (OB0 → OB15) and nearby forest soil (NF) were analyzed with respect to different physicochemical variables in triplicates. Microsoft Excel-97 was used for statistical processing of data. Soil analyses data were subjected to simple correlation analysis to test the level of significance among soil attributes using SPSS (Version 17.0). The stepwise multiple regression analysis was used to quantify the contribution of physicochemical soil variables influencing the variability in microbial biomass pool over time across the sites using Minitab 16 software. The principal component analysis was performed using Statistrix PC DOS Version- 2.0 (NH Analytical software). The redundancy analysis (RDA) was performed using Microsoft Excel XLSTAT-2014 (Version 2.03).

3. RESULTS

3.1 Physicochemical characterization

The clay content, hydrological regimes and pH of six different age series coal mine overburden spoil (OB0 → OB15) as well as nearby forest soil was presented (Table 1). The analysis indicated gradual increase in clay percentage from OB0 (5.3%) to OB15 (11.8%) over time, which was found to be statistically significant (r = 0.979, $p<0.001$). The clay percentage exhibited by nearby NF soil (12.9%) revealed 2.43 folds higher compared to OB0. The water holding capacity exhibited an increasing trend from OB0 (26.73%) to OB15 (45.36%), which was marked to be statistically correlated with the age of mine overburden spoil (r = 0.946, $p<0.001$). Similar trend was also exhibited by moisture content across the sites with minimum in OB0 (6.913%) and maximum in OB15 (10.238%) over time (r = 0.993, $p<0.001$). However, the bulk density showed a decline trend from OB0 (1.712 g/cm^3) to OB15 (1.268 g/cm^3) over time, which was found to be statistically significant (r = 0.926, $p<0.001$). The bulk density, water holding capacity and moisture content in nearby NF soil was found to be 1.236%, 47.13% and 11.319% respectively (Table 1). The pH of mine spoil was found to be within the acidic range (6.12 to 6.68) with minimum in OB0 and maximum in OB15 over time. The study suggested that pH of mine spoil progressively increase towards the neural range with the increase in age of mine overburden spoil (Table 1).

Table 1. Clay percentage, Bulk density (BD), water holding capacity (WHC), moisture content (MC) and pH of coal mine spoil collected from different age series overburdens (OB0 → OB15) and NF soil from (0-15) cm soil depth.

Parameters	Coal mine spoil from different age series overburdens from (0-15) cm soil depth						NF soil
	OB0	OB3	OB6	OB9	OB12	OB15	
Clay (%)	5.3 ± 0.15	7.5 ± 0.21	9.1 ± 0.33	10.3 ± 0.19	11.2 ± 0.42	11.8 ± 0.29	12.9 ± 0.36
BD (g/cm^3)	1.712 ± 0.035	1.584 ± 0.028	1.389 ± 0.032	1.321 ± 0.033	1.293 ± 0.024	1.268 ± 0.022	1.236 ± 0.025
WHC (%)	26.73 ± 1.278	33.29 ± 0.946	39.13 ± 1.214	42.45 ± 1.379	44.67 ± 0.884	45.36 ± 1.169	47.13 ± 1.294
Moisture (%)	6.913 ± 0.195	7.328 ± 0.294	7.967 ± 0.218	8.672 ± 0.254	9.547 ± 0.284	10.238 ± 0.372	11.319 ± 0.343
pH	6.12 ± 0.05	6.21 ± 0.04	6.35 ± 0.06	6.42 ± 0.04	6.59 ± 0.05	6.68 ± 0.04	6.92 ± 0.03

Values are mean ± SD; (n = 5). *$p<0.001$ level of significance.

3.2 *Organic C, Total N and Extractable P*

The study indicated that organic carbon, total nitrogen and extractable phosphorus content in OB0was beyond the detectable limit (Table 2). Wide variation in organic carbon (0.358 ± 0.035 to 2.684 ± 0.227 mg C/g spoil), total nitrogen (32.963 ± 3.315 to 1267.25 ± 41.136 µg N/g spoil) and extractable phosphorus (6.359 ± 0.638 to 171.152 ± 11.532µg P/g spoil) was exhibited by different age series coal mine overburden spoil with minimum in OB2 and maximum in OB15 over time across the sites (Table 2). The analysis of variance showed that there was gradual increase in organic C ($r = 0.992$, $p<0.001$), total N ($r = 0.952$, $p<0.001$) and extractable P ($r = 0.939$, $p<0.001$), which was observed to be statistically significant with the increase in age of the mine spoil. However, the nearby NF soil exhibited relatively higher organic C (3.705 ± 0.264 mg C/g spoil), total nitrogen (1733.12 ± 32.576 µg N/g spoil) and extractable phosphorous (272.531 ± 13.537 µg P/g spoil) compared to different age series coal mine overburden spoil (Table 2).

Table 2. Organic carbon, total nitrogen and extractable phosphorous content in coal mine spoil samples collected from different age series overburdens (OB0 → OB15) and nearby NF soil from (0-15) cm soil depth.

Soil Profiles	Organic Carbon (mg C. g^{-1} spoil)	Total Nitrogen (µg N. g^{-1} spoil)	Extractable Phosphorous (µg P. g^{-1} spoil)
OB0	nd*	nd*	nd*
OB3	0.358 ± 0.035	32.963 ± 3.315	6.359 ± 0.638
OB6	1.118 ± 0.051	83.562 ± 13.968	14.137 ± 1.534
OB9	1.634 ± 0.132	335.523 ± 21.425	54.522 ± 3.382
OB12	2.118 ± 0.219	915.658 ± 39.559	108.452 ± 13.658
OB15	2.684 ± 0.227	1267.25 ± 41.136	171.152 ± 11.532
NF soil	3.705 ± 0.264	1733.12 ± 32.576	272.531 ± 13.537

Values are mean ± SD; (n = 5).nd*: beyond detectable limit.

3.3 *Microbial biomass pool*

Comparative assessment of six different age series coal mine overburden spoil in chronosequence (OB0 → OB15) and nearby NF soil was performed in order to differentiate soil profiles based on their microbial biomass pool size. The study indicated that the MB-C, MB-N and MB-P in OB_0 were beyond the detectable limit (Table 3). It is evident form the study that there was gradual improvement in MB-C (14.357 ± 2.561 to 596.358 ± 23.596 µg C/g spoil), MB-N (1.983 ± 0.113 to 93.453 ± 8.352 µg N/g spoil) and MB-P (1.935 ± 0.348 to 48.543 ± 2.993 µg P/g spoil) pool size from the nutrient deficient OB2 to OB15 over a span of 15 years (Table 3). The progressive increase in MB-C ($r = 0.944$, $p<0.001$), MB-N ($r = 0.941$, $p<0.001$) and MB-P ($r = 0.930$, $p<0.001$) over time across the sites was found to be significant (Figure 1a-c). Further, the nearby NF soil exhibited relatively higher MB-C (947.564 ± 31.572 µg C/g spoil), MB-N (141.288 ± 6.266 µg N/g spoil) and MB-P (48.543 ± 2.993 µg P/g spoil) level compared to different age series coal mine overburden spoil.

Table 3. Microbial biomass-C, microbial biomass-N and microbial biomass-P in coal mine spoil samples collected from different age series overburdens (OB0 → OB15) and nearby NF soil from (0-15) cm soil depth.

Soil Profiles	MB-C (μg C. g^{-1} spoil)	MB-N (μg N. g^{-1} spoil)	MB-P (μg P. g^{-1} spoil)
OB0	nd*	nd*	nd*
OB3	14.357 ± 2.561	1.983 ± 0.113	nd*
OB6	65.436 ± 6.865	5.364 ± 0.852	1.935 ± 0.348
OB9	173.554 ± 11.527	23.365 ± 3.452	8.618 ± 0.594
OB12	421.982 ± 9.654	65.613 ± 2.769	17.549 ± 0.783
OB15	596.358 ± 23.596	93.453 ± 8.352	29.667 ± 3.241
NF soil	947.564 ± 31.572	141.288 ± 6.266	48.543 ± 2.993

Values are mean ± SD; (n = 5).nd*: beyond detectable limit.

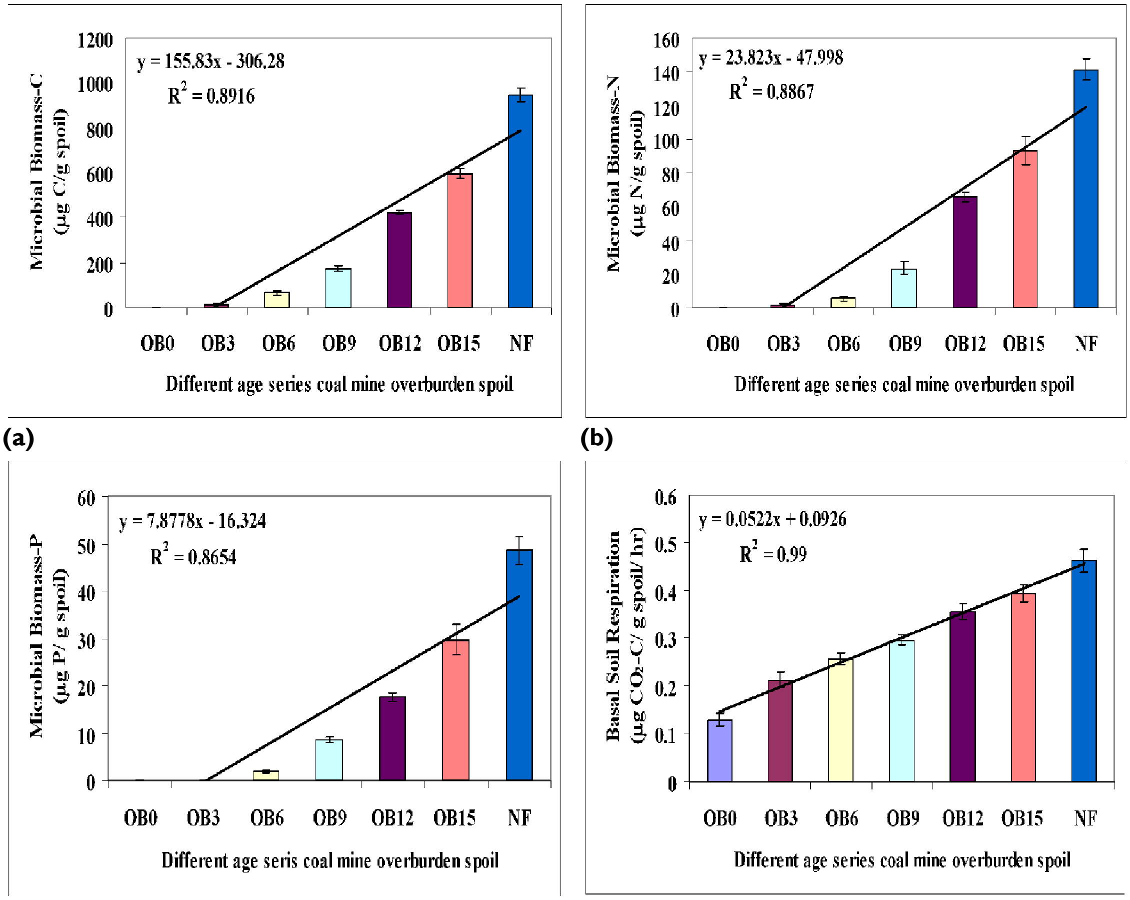
3.4 Microbial basal soil respiration

The microbial basal soil respiration rate and its fluctuations in different age series coal mine overburden spoil over time were presented (Table 4). Significant variation in basal soil respiration rate was observed with minimum on OB0 (0.129 ± 0.013 µg CO_2-C/g spoil/h) and maximum in OB15 (0.394 ± 0.019 µg CO_2-C/g spoil/h). The study indicated consistent increase in BSR with the increase in age of the mine spoil, which was found to be statistically significant (r = 0.994, $p<0.001$) (Figure 1d). Besides, the BSR exhibited by nearby NF soil (0.463 ± 0.0.25 µg CO_2-C/g spoil/h) was found to be relatively higher compared to six different age series coal mine overburden spoil in chronosequence across the sites (Table 4).

Table 4. Basal soil respiration rate in different age series coal mine spoil (OB0 → OB15) and nearby NF soil from (0-15) cm soil depth.

Parameter	Coal mine spoil from different age series overburdens from (0-15) cm soil depth						NF soil
	OB0	OB3	OB6	OB9	OB12	OB15	
BSR (µg CO_2-C/g spoil/h)	0.129 ± 0.013	0.213 ± 0.016	0.258 ± 0.012	0.297 ± 0.011	0.356 ± 0.016	0.394 ± 0.019	0.463 ± 0.025

Values are mean ± SD; (n = 5).



(a) (b)

(c) (d)

Values are mean ± SD; (n = 5).

Fig 1(a-d). Wide variation in (a) Microbial biomass-C, (b) Microbial biomass-N, (c) Microbial biomass-P and (d) Basal soil respiration in different age series coal mine overburden spoil (OB0 → OB15) and nearby NF soil.

3.5 *Integrating quotients*

The analysis indicated consistent increase in different integrating quotients such as MB-C:OC (4.010 to 22.219), MB-N:TN (6.015 to 8.152) and MB-P:EP (13.687 to 17.333) with minimum on OB0 and maximum in OB15 over time across the sites (Table 5). However, the MB-C:OC (25.575), MB-N:TN (8.152) and MB-P:EP (17.811) in nearby NF soil was found to be relatively higher compared to different age series mine spoil.

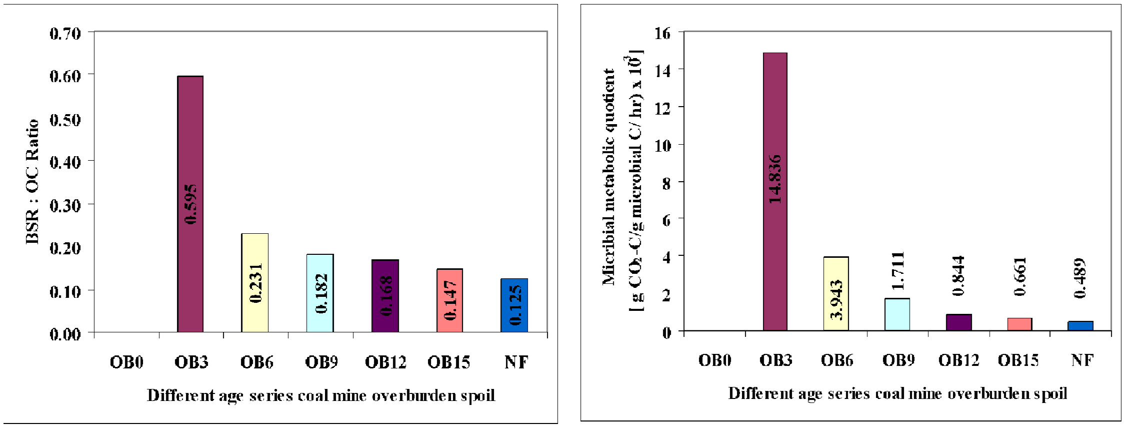
Table 5. Different integrating quotients exhibited by mine spoil samples collected from different age series overburdens (OB0→ OB15) and nearby NF soil.

Parameter	Coal mine spoil from different age series overburdens from (0-15) cm soil depth						NF soil
	OB0	OB3	OB6	OB9	OB12	OB15	
MB-C:OC	nd*	4.010	5.852	10.621	19.923	22.219	25.575
MB-N:TN	nd*	6.015	6.419	6.963	7.165	7.374	8.152
MB-P:EP	nd*	nd*	13.687	15.806	16.181	17.333	17.811

nd*: beyond detectable limit.

3.6 *Microbial metabolic quotients*

The BSR:OC ratio revealed a decline trend with maximum in OB3 (0.595) and minimum in OB15 (0.147) with the increase in age mine spoil (Figure 2a). Lower level of BSR:OC ratio was estimated in nearby NF soil (0.125) compared to different age series coal mine spoil. Besides, the microbial metabolic quotient (qCO_2) was calculated using the mean value of basal soil respiration and microbial biomass carbon in different age series mine spoil (OB0 → OB15) and nearby NF soil. The analysis indicated that the microbial metabolic quotient was found to be maximum in OB3 (14.836×10^{-3} g CO_2-C/g microbial C/h) and minimum in OB15 (0.661×10^{-3} g CO_2-C/g microbial C/h) over time (Figure 2b). However, the qCO_2 exhibited by OB0 was found to be beyond the detectable limit. The study clearly suggested that the microbial metabolic quotient exhibited a decline trend from OB3 to OB15 with the increase in age of mine overburden spoil. Further, the qCO_2 exhibited by nearby NF soil (0.489×10^{-3} g CO_2-C/g microbial C/h) was found to be relatively less compared to different age series coal mine spoil across the sites (Figure 2b).



(a) **(b)**

Fig 2 (a-b). Wide variation in (a) BSR:OC ratio and (b) microbial metabolic quotient (qCO_2) in different age series coal mine overburden spoil (OB0 → OB15) and nearby NF soil across the sites.

4. Discussion

The progressive increase in clay percentage among six different age series coal mine overburden spoil in chronosequence (OB0 → OB15) may be due to the accumulation of available nutrients supported by gradual establishment of vegetational development over time.[1,12] Gradual improvement in clay percentage was reported to stabilize and restore the disturbed habitat[2,3,4] by enhancing microbial amelioration[44] and thereby promoting soil structural stability and aggregation[1,23,45] with increase in age of coal mine overburden spoil across the sites. Textural composition and particle size distribution influence different hydrological regimes such as bulk density, water holding capacity and moisture content, which in turn regulates space, air and water availability for microbial amelioration. The decline in bulk density can be interpreted as the reduction in soil compactness because of the development of soil micropore space with the increase in age of mine overburden spoil. The clay fraction has an ultimate bearing on bulk density,[12] which is influenced by land use patterns and soil types.[46] Relatively higher bulk density in OB0 may be attributed to lower clay content and devoid of vegetation, whereas the reduced bulk density in OB25 with closed resemblance with the nearby NF soil, which may be due to the gradual accumulation of clay that promote macroaggregation and organic matter input supported by vegetational cover over time. The negative correlation between bulk density and organic carbon (r = -0.904, $p<0.01$) in different age series coal mine overburden spoil over time substantiated the concept.[1,23] The water holding capacity and moisture content progressively increase from OB0 to OB15, which may be due to gradual establishment of vegetation over time, positive influence of canopy that prevent loss of water through evaporation by not allowing direct exposure of soil surface to incoming radiation.[1,4] Besides, soil organic matter is also reported to be key components influencing different hydrological regimes.[14] It is evident from the study that WHC exhibited positive correlation with organic C (r = 0.922, $p<0.01$), total N (r = 0.808, $p<0.05$), and extractable P (r = 0.785, $p<0.05$). Moisture content also exhibited positive correlation with organic C (r = 0.996, $p<0.01$), total N (r = 0.978, $p<0.01$), and extractable P (r = 0.971, $p<0.01$) (Table 6). Relatively higher WHC and MC in nearby NF soil may be due to gradual establishment of vegetation and accumulation of organic C associated with clay fraction over time.[13,14]Being site-specific, soil pH is often modeled as positive linear relationship for the assessment of soil quality, which is an additional criterion used for soil classification and mapping.[20] The pH of different age series mine spoil was found to be in acidic range (6.12 to 6.68), which may be due to mineral depositions,[1,23] accumulation of organic C, formation of organic acids and oxidation of residual elements that hinders the release of plant nutrients.[23] Improvement in soil pH towards neutrality may be due to restoration by natural succession or vegetational pattern and promotion of organic matter decomposition over time.

Table 6. Simple correlation coefficient (*r*) between different soil properties among six different ages series coal mine overburden spoil (OB0 → OB15).

Soil attributes	Clay	BD	WHC	MC	pH	OC	TN	EP	MBC	MBN	MBP	BSR
Clay	1											
BD	-0.978^{**}	1										
WHC	0.991^{**}	-0.995^{**}	1									
MC	0.952^{**}	-0.883^{**}	0.907^{**}	1								
pH	0.953^{**}	-0.885^{**}	0.908^{**}	0.997^{**}	1							
OC	0.962^{**}	-0.904^{**}	0.922^{**}	0.996^{**}	0.997^{**}	1						
TN	0.874^{**}	-0.773^{*}	0.808^{*}	0.978^{**}	0.971^{**}	0.960^{**}	1					
EP	0.859^{**}	-0.751^{*}	0.785^{*}	0.971^{**}	0.969^{**}	0.960^{**}	0.990^{**}	1				
MBC	0.865^{**}	-0.760^{*}	0.795^{*}	0.975^{**}	0.974^{**}	0.963^{**}	0.994^{**}	0.998^{**}	1			
MBN	0.859^{**}	-0.751^{*}	0.788^{*}	0.973^{**}	0.969^{**}	0.957^{**}	0.997^{**}	0.997^{**}	0.999^{**}	1		
MBP	0.845^{**}	-0.736^{*}	0.769^{*}	0.964^{**}	0.963^{**}	0.954^{**}	0.986^{**}	0.999^{**}	0.997^{**}	0.995^{**}	1	
BSR	0.986^{**}	-0.932^{**}	0.955^{**}	0.985^{**}	0.987^{**}	0.988^{**}	0.938^{**}	0.928^{**}	0.934^{**}	0.929^{**}	0.918^{**}	1

**** Correlation is significant $p < 0.01$ and * correlation is significant $p < 0.05$.**

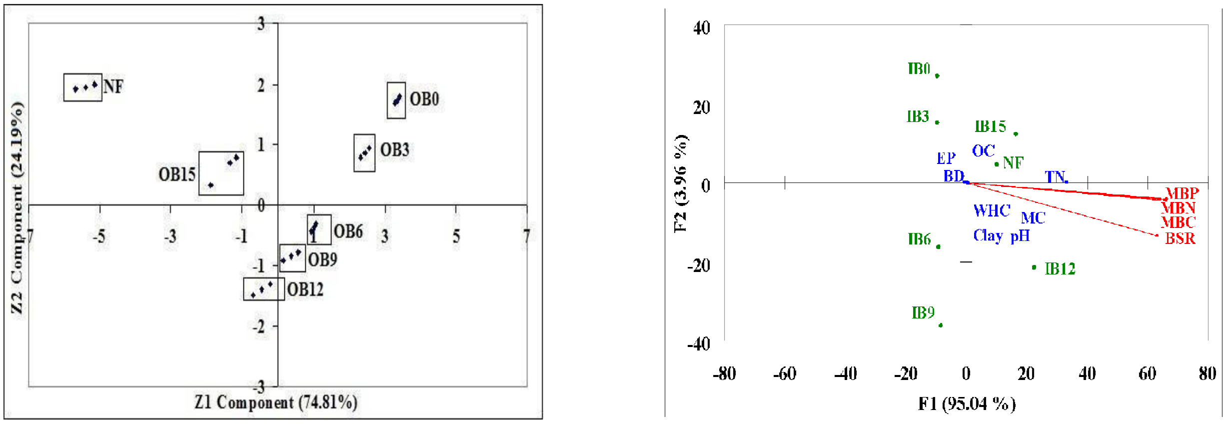
The consistent improvement in organic C, total N and extractable P in different age series mine spoil reflect the sign of restoration of coal mine spoil over time.[1,16,47] The study indicated gradual increase in organic C, total N and extractable P from the nutrient deficient coal mine overburden spoil to enriched NF soil, which may be due to the gradual establishment of vegetation cover, input of litter from vegetation compartment and associated decomposition during the course of passive or active restoration.[4,48,49]The variation in organic C exhibited positive correlation with the increase in clay percentage (r = 0.962, $p<0.01$) across the sites (Table 6), which suggested that minimal difference in clay percentage alters the level of organic C accumulation in reclaimed mine spoil.[50,51] Besides, the variation of soil organic C associated with primary soil particle depends on the land use patterns, which is reported to promote soil aggregation, structural stability and nutrient retention, hence considered as reliable indicator for the assessment of nutritional status.[12,47,52] Significant variation in total N and extractable P in different age series coal mine overburden spoil over time was revealed by the simple correlation analysis (Table 6). Gradual increase in total N from nutrient deficient mine spoil to enriched NF soil may be due to the input from plant species capable of nitrogen fixing potential through the development of mycorrhiza and other nutrient immobilizing microbial colonization, which contribute to soil quality both in managed and natural ecosystems.[12,20] Besides, the minimal extractable P in OB0 may be due to its acidic nature, which restricts microbial growth and amelioration, mineralization and organic matter decomposition.[19] Nevertheless, the variation in organic C in different age series coal mine overburden spoil was found to be positively correlated with total N (r = 0.960, $p<0.01$) and extractable P (r = 0.960, $p<0.01$) over time (Table 6). Wide variation in MB-C, MB-N and MB-P was exhibited by different age series coal mine spoil, which showed an increasing trend from OB0 to OB15 with the increase in age of mine overburden spoil. Besides, OB0 represents nutrient deficient with altered geomorphic system inhibiting vegetation development and microbial growth due to minimal organic C, total N, extractable P and poor hydrological regimes, which inferred that the size of microbial biomass pool in OB0 was below the detectable limit. Comparative analysis of microbial biomass data revealed greater proliferation of MB-N over MB-C and MB-P over a period of 12 years, which reflects relatively faster nitrogen immobilization that support mine spoil genesis over time across the sites.[16] Greater proliferation of microbial biomass pool plays an important role in pedogenesis and amelioration of microclimatic conditions in degraded ecosystem.[26] Contribution of microbial biomass towards nutrient flow, organic matter turnover and soil structural stability have been reported by microbial ecologists and hence can be used as ecological biomarker for the assessment of mine spoil genesis.[20,28,53,54]The basal soil respiration exhibited consistent improvement (0.129 to 0.394 µg CO_2-C/g spoil/hr) in different age series coal mine overburden spoil over time across the sites. Low basal soil respiration rate in OB0 evidences low microbial turnover compared to OB15 due to low substrate availability required for microbial growth and the exposure of environmental extremities (desiccation, temperature, metal contaminants). However, the gradual inputs of organic C from vegetational component promote the level of BSR in OB15 over time. BSR exhibited positive correlation with MB-C (r = 0.934, $p < 0.01$), MB-N (r = 0.929, $p < 0.01$), MB-P (r = 0.918, $p < 0.01$) and OC (r = 0.988, $p < 0.01$) respectively (Table 6). The pace and progress of mine spoil genesis was determined by different integrating quotients such as MB-C:OC, MB-N:TN and MB-P: EP. Ratio of microbial biomass nutrient to soil nutrient reflects the quantum of nutrients available in microbial biomass pool, which can be served as integrative quotients for monitoring mine spoil genesis. The MB-C:OC ratio provides an insight into soil organic C status, which is proposed to be the functional index of soil subsystems.[22]The time dependent increase in MB-C:OC from OB3 (4.010) to OB15 (22.219) indicated the shift in carbon mineralization rate due to the decline in metal contaminants with the gradual accumulation of organic C over time. The variation in organic C was found to be positively correlated with MB-C (r = 0.963, $p<0.01$) across the sites (Table 6), which corroborates with earlier findings.[22,28] Besides, the greater influx of organic C to microbial biomass leads to more influx of N and P.[32] The MB-N:TN ratio exhibited an increasing trend from OB3 (6.015) to OB15 (7.374) over time. Similarly, the quantum of extractable P reflected in MB-P ranged from OB6 (13.687) to OB25 (17.333), which substantiates the role of microbial biomass for phosphorous immobilization and can be considered as the critical limiting nutrient that impede the progress of mine spoil genesis. The minimal value of different integrating quotients exhibited by OB0 may be due to the fact that the microbes are under stress in metal contaminated mine spoil and less efficient for organic C utilization.[28] The time dependent decline in BSR:OC ratio from OB3 (0.595) to OB15 (0.147) across the sites substantiated the fact that the higher value of BSR:OC ratio confirms the greater use of organic C by the existing microbial community in OB0 compared to different age series coal mine overburden spoil, which revealed the elevated stress condition of the habitat.[28] Besides, the microbial metabolic quotient (qCO$_2$) reflects the efficacy of microbial community structure with reference to their substrate/energy utilization,[32,55] which can be used as an ecological biomarker for microbial mediated C mineralization in turn for monitoring mine spoil genesis.[22,28] Relatively higher qCO$_2$ in OB0 may be due to the lack of organic C and presence of microbial community with 'r'- strategy ecotype known for lower C mineralization, whereas minimum qCO$_2$ in OB15 may be explained on the basis of the presence of complex detritus and more efficient microbial community with 'k'- strategy ecotype.[28,56] More labile C that is readily decomposable would favour the opportunistic 'r'-strategy ecotype over the enzyme procedures 'k'-strategy ecotype.[57] Therefore, 'r'-strategy ecotype represents the disturbed habitat whereas 'k'-strategy ecotypes shows the recovery of the disturbed habitat associated with low qCO$_2$.[28,58,59] Thus, the decline in qCO$_2$ from OB0 to OB15 with the increase in age of mine overburden spoil strongly reflects the progress of mine spoil genesis. The stepwise multiple regression analysis was performed to quantify the contribution of different soil properties influencing microbial biomass pool as dependent variable[60] in different age series coal mine overburden spoil over time (Table 7). The analysis indicated that 79.8% of the variability in MB-C was explained by OC, additional 19.4% by clay as 2nd variable and a marginal effect by MB-N (0.7%). Besides, the variability in MB-C is correlated with MB-N (99.7%, $p<0.001$) and a marginal effect by EP as 2nd variable. About 84.1% variability in MB-N was explained by MB-C. The 2nd and 3rd variables of importance were TN (12.3%) and OC (3.5%) respectively (Table 7). With EP as 1st variable explained 99.8% of the variability in MB-P and a marginal effect by clay as 2nd variable. About 87.5% of the variability in BSR was explained by OC. The 2nd and 3rd variables of importance explaining the variability in BSR were clay (8.9%) and MB-C (3.5%) respectively (Table 7). With clay as 1st variable explained 89.4% of the variability in BSR, additional 6.1% by MB-C and 4.2% by MC as 2nd and 3rd variables, and a marginal effect by TN as 4th variable.

Table 7. Stepwise multiple regression analysis revealed the contribution of different soil attributes on the variability in microbial biomass pool across the sites

Parameter	Equation(s)	R^2*
MB-C	= 105.2139 + 15.86 OC	0.798
	= 0.1938 + 0.05 OC + 7.47 Clay	0.992
	= 0.1659 + 0.086 OC + 0.0321 MB-N	0.999
	= 8.872 + 6.52 MB-N	0.997
	= 6.309 + 3.66 MB-N + 1.53 EP	0.999
MB-N	= -1.2500 + 0.1531 MB-C	0.841
	= -1.7183 + 0.0952 MB-C + 0.0302 TN	0.964
	= 0.2575 + 0.1050 MB-C + 0.0318 TN – 3.68 OC	0.999
MB-P	= -0.8808 + 0.1793 EP	0.998
	= 1.4939 + 0.1911 EP – 11.4 Clay	0.999
BSR	= 0.15883 + 0.0859 OC	0.875
	= 0.02564 + 0.0459 OC + 0.0205 Clay	0.964
	= -0.0800 – 0.0381 OC + 0.400 Clay + 0.00017 MB-C	0.999
	= -0.01364 + 0.0159 Clay	0.894
	= -0.02645 + 0.0304 Clay + 0.00010 MB-C	0.955
	= 0.26986 + 0.0446 Clay + 0.0025 MB-C – 0.054 MC	0.997
	= 0.41837 + 0.0508 Clay + 0.00023 MB-C – 0.081 MC + 0.00005 TN	0.999

***All R^2- values are significant at $p < 0.001$.**

Further, the principal component analysis was performed to discriminate six different age series coal mine overburden spoil and nearby NF soil based on their physicochemical properties and microbial biomass pool size.[61,] The analysis indicated that Z1 and Z2 components explained maximum variance with respect to different soil attributes and their cumulative percentage of variance was found to be 99%, which can able to segregate different soil profiles into independent clusters (Figure 3a). Further, the redundancy analysis (RDA) was performed to explain the relationship between different soil variables altogether and quantify their contribution towards the shift in microbial biomass pool and basal soil respiration rate in different age series coal mine spoil (OB0 → OB15) over time and nearby NF soil across the sites (Figure 3b). RDA analysis revealed that the different age series mine spoil had distinctly different microbial community structure, and the changes in microbial biomass pool and basal soil reparation rate is influenced by the shift in different physicochemical soil attributes that alter the microenvironment and efficiency of readily mineralizable resources by soil microbes. The RDA analysis provides an insight into the multifaceted nature of these soil variables that shape the microbial community structure in chronosequence coal mine overburden spoil over time.[62]



(a) **(b)**

Fig 3(a-b). (a) Principal component analysis revealed the segregation of six different age series mine overburden spoil (OB0 → OB15) and nearby NF soil into independent clusters; (b) RDA analysis of different age series mine overburden spoil (OB0 → OB15) and nearby NF soil revealed highest scores on each of the first two axes with site codes.

The study suggested that the different soil attributes revealed gradual improvement in different age series coal mine overburden spoil over time, which indicated the progress of mine spoil genesis. Considering the soil features of nearby NF soil as unit, the proportionate level of parameters for different mine overburden spoils were calculated. Further, attempt was made to calculate the time period required for the restoration of OB_0 to reach the soil features of nearby NF soil. Accordingly, the positive correlation was observed between soil features and age of mine overburden spoil ($r = 0.960$; $p < 0.01$), which explained 92.21% variability in soil features with the increase in age of mine overburden spoil (Figure 4). Taking the value of the nearby NF soil (*i.e.* 1) as 'X', the equation was used to calculate the age of the nearby NF soil *i.e.* 29.1468 years. The study suggested that OB0 to attain the soil features of nearby NF soil through mine spoil genesis shall take approximately 29.15 years provided the mine spoil is not subjected to any other interferences.

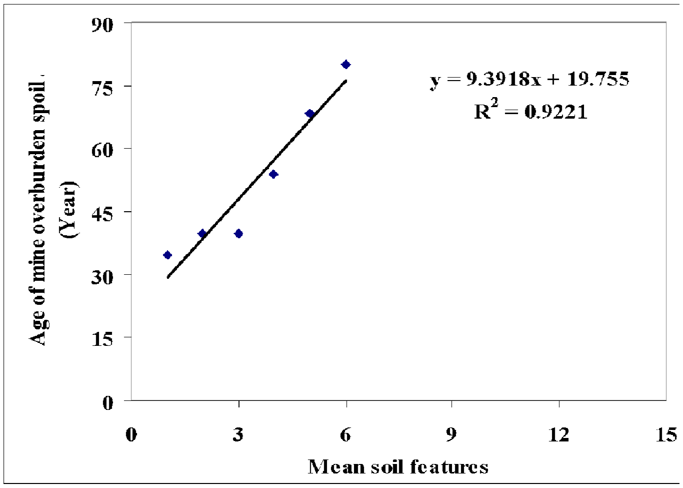


Fig 4. Relationship between mean soil features with the age of six different coal mine overburden spoil in chronosequence (OB0 → OB15) across the sites

Gradual improvement in clay, hydrological regimes, soil pH organic C, total N and extractable P over time clearly demonstrated their C sink potential across the sites. The MB-C, MB-N and MB-P exhibited an increasing trend from OB0 to OB15 over time. Time dependent improvement in integrating quotients such as MB-C:OC, MB-N:TN, MB-P: EP and BSR:OC reflect the sign of mine spoil genesis. Decline in qCO_2 with the increase in age of mine spoil strongly reflects the progress of mine spoil genesis. Gradual proliferation of microbial biomass pool contributed by physicochemical properties over time support nutrient flow, organic matter turnover and structural stability of mine spoil, which can be used as potential biomarker for monitoring mine spoil genesis.

5. CONCLUSION

The gradual proliferation of biomass pool contributed by different physicochemical properties over time support nutrient flow, organic matter turnover and structural stability in the chronosequence coal mine spoil, which reflects the sign of ecological restoration and hence can be used as potential biomarker of monitoring mine spoil genesis. It is evident from the study that the mine spoil of OB0 shall take approximately 29.15 years to attain the soil features of the nearby NF soil through the process of mine spoil genesis. The study clearly indicated that the quantum of available nutrient reflected in the microbial biomass pool determines the pace and progress of mine spoil genesis, and therefore the periodic assessment of microbial biomass pool in degraded ecosystem can be useful for designing appropriate management strategies for sustainable ecosystem.

5. Authors Contribution statement

Ms. Payal Agrawal performed the experiments, gathered the data with regards to different soil attributes. Dr. Jitesh Kumar Maharana analyzed the data through statistical analysis and necessary inputs towards designing the manuscript. Dr. Amiya Kumar Patel along with other authors discussed the methodology and compilation of the results to fulfill the objectives of the investigation.

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7. Conflict of interest

Conflict of interest declared none.

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