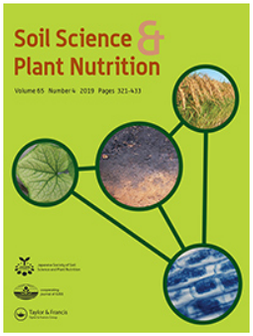

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ORIGINAL ARTICLE

Microbial community structure and relationship with physicochemical properties of soil stockpiles in selected South African opencast coal mines

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ABSTRACT

At present, there is no comprehensive soil quality assessment practice for soil stockpiles in the South African coal mining industry. Soil microorganisms and enzymes are suitable indicators for soil quality monitoring. Therefore, this study investigated the microbial community and enzyme (beta-glucosidase and urease) activities in soil stockpiles of opencast coal mines in the coal-rich region of South Africa. Soil stockpiles of three opencast coal mines were sampled at depths of 0–20 cm ('topsoil') and >20 cm ('subsoil') across three seasons. Beta-glucosidase and urease activities were mostly higher in soil stockpiles than in unmined soils and were significantly influenced ($P < 0.05$) by the interaction of site and seasonal factors. However, analyses of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) profiles of partial 16S rRNA gene and internally transcribed spacer 2 (ITS2) sequences revealed higher microbial diversity in unmined (reference) soils compared to soil stockpiles across all seasons. Redundancy analysis further revealed that microbial communities of topsoil were not significantly ($P > 0.05$) influenced by soil properties, whereas microbial communities of subsoils were significantly ($P < 0.05$) influenced by pH, organic carbon, total nitrogen and phosphorus contents. Furthermore, operational taxonomic units (OTUs) belonging to genera of known phytobeneficial species such as *Azomonas, Aureobasidium, Phialocephala, Phoma* and *Sordariomycetes* were detected in these soils. Overall, results suggest that the microbial community structure and diversity observed in stockpiles is impaired (compared to the unmined site), although variations in the microbial community structure of soil stockpiles across seasons are site-specific. The impaired microbial community of stockpiles may have negative implications on soil biological processes driven by microbes; especially those that are critical for nutrient cycling and ecosystem sustainability. More importantly, such alteration in soil biodiversity may impair post-mining land use capability of stockpile soils.

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Coal mining; enzyme activities; bacteria; fungi; soil microorganism; stockpiling

1. Introduction

The soil is a non-renewable resource which plays crucial roles that are paramount to human existence and sustainability of other ecosystems (Faber *et al.* 2013). Often, this non-renewable resource is disturbed through several anthropogenic activities, including agriculture and mining. The coal deposits (largely bituminous) in South Africa are mostly below arable soils, especially in the Mpumalanga Highveld portion of the grassland biome area. This area provides important ecosystem services such as provisioning services (e.g., arable use) and cultural services (recreational). Unfortunately, surface (opencast) mining of these coal deposits contributes to the loss of such important ecosystem services. Therefore, the rehabilitation of mined lands towards restoring pre-disturbance ecosystem services or achieving an acceptable post-mining land use capability is paramount.

For the post-mining restoration of the soil profile and ecosystem services, mining regulations stipulate that the original topsoil and reusable horizons – the nutrient-rich and plant-growth supporting horizons – must be carefully excavated and stockpiled (stored) separately from other overburden materials until post-mining rehabilitation (Strohmayer 1999). In most cases, the soil is stored for several years, during which changes in the quality of the soil may occur (Strohmayer 1999). Unfortunately, these changes may contribute to the impairment of important soil health components, including physico-chemical properties and diversity of the soil biota (Harris *et al.* 1989; Cardoso *et al.* 2013; Ezeokoli *et al.* 2019). Previous studies have established that the stockpiling operations contribute to the hard-setting of soil, increased soil compaction levels, poor soil structure, reduction in soil water-holding capacity, decreased soil biomass, loss of nutrients and poor overall quality of topsoil (Abdul-Kareem and McRae 1984; Ghose 2004; Coetzee 2016; Paterson *et al.* 2019). Logically, the quality of stockpiled soil is linked to the success of post-mining rehabilitation because the soil is reapplied during rehabilitation and prior to revegetation (Sheoran *et al.* 2010). Hence, an assessment of the quality of soil stockpiles in coal

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Supplemental data for this article can be accessed here.

mines will provide an early forecast of the likelihood of post-mining land rehabilitation success or otherwise.

For soil health assessments, a combination of physical, chemical, and biological components of the soil are important parameters. Such an assessment provides a comprehensive insight into the condition of the soil (Doran and Parkin 1994; Claassens *et al*. 2008, 2012). With regard to soil biological components, microbes and enzymes play several important roles in the soil ecosystem. Such roles include the mobilization of essential nutrients in the soil, mediation of plant nutrient uptake, plant-pathogen resistance, secretion of plant growth-promoting hormones and decomposition of soil organic matter (Tinker 1984; van Veen and Kuikman 1990; van der Heijden *et al*. 2008; Adeleke *et al*. 2012, 2017). Hence, enzyme activities and diversity of microbes have been used as soil quality indices for ascertaining nutrient cycling and availability in the soil (Tabatabai and Dick 2002). Such indices are also used for assessing changes in soil organic matter (Six *et al*. 2004) and the impacts of farming practices (García-Ruiz *et al*. 2009), soil management (Bending *et al*. 2004), tillage (Fließbach *et al*. 2007), plant genotype (van Wyk *et al*. 2017) and fertilizer application (Mandal *et al*. 2007). Generally, higher soil microbial diversity and activity suggest the functional plasticity or stability of the soil ecosystem to external constraints.

At present, there is no comprehensive soil health assessment practice for soil stockpiles in the South African coal mining industry. It has been previously established that soil microorganisms and enzymes are sensitivity to anthropogenic disturbances (Dose *et al*. 2015; Nkuekam *et al*. 2018). However, there is still a knowledge gap especially as this relates to the extent at which microbial communities and enzyme activities in soil stockpiles are influenced across climatic seasons. In the present study, we tested two hypotheses viz (1.) that enzyme activities and microbial diversity of topsoil stockpiles (disturbed) are impaired compared to adjacent unmined (undisturbed) soils, and (2.) that microbial communities and enzyme activities in soil stockpiles vary across seasons. To achieve this, the present study explored the microbial community structure and dynamics, as well as activities of selected enzymes of soil stockpiles in three South African coal mines and an unmined reference site across three seasons. By making comparisons of these parameters between reference soils and soil stockpiles, we aimed to provide some indication of the health status of soil stockpiles as well as appropriateness of current soil stockpiling processes in the South African coal industry.

2. Materials and methods

2.1. Study sites and soil sampling

Three opencast coalmines (designated A, B, and C) located in the coal-rich eMalahleni area (24°0′–27°30′ S, 28°15′–32°5′ E) of the Mpumalanga Province of South Africa, were selected for this study (exact locations of mines and names are withheld due to a confidentiality agreement). These mines are actively being mined for coal (bituminous thermal grade coal). Mine B is approximately 48 km from mine C, while mine A was 160 km from mine B and C. The climate of the eMalahleni area is usually warm and moist in the summer, while the winter season is usually cold and dry with frost. This area receives about 750 mm of rainfall annually, 85% of which occurs during the growing season (October to March) (ARC, 2016). The vegetation across all sites was predominantly grass species (*Digitaria eriantha, Cynodon dactylon* and *Eragrostis curvula*) with an estimated basal cover of 10% – 35% across soil stockpiles. The soil stockpiles at the time of sampling were sparsely vegetated (~10% grass and ~2% forbs cover) and have been stored for at least 5 years. The age of stockpiles was not considered in this study due to possible differences in mining practices as previously reported in a parallel study (Ezeokoli *et al*. 2019). Unmined lands adjacent to mine A (approx. 200–500 m distant) served as 'reference'. The plant cover (grass species) of the reference site was approximately 60% and predominantly comprised *Digitaria eriantha* and *Cynodon dactylon*. At the time of sampling, the reference site was not being utilized for any anthropogenic activities (especially mining). However, due to the proximity of the reference site to infrastructures such as roads and settlements, total absence of anthropogenic influences cannot be excluded.

Soil sampling was conducted during the summer (February), winter (July), and spring (September) seasons of 2015. At each study site, a minimum of five soil stockpiles was randomly sampled per season at depths of 0–20 cm (hereafter referred to as 'topsoil') and >20 cm (hereafter referred to as 'subsoil'), respectively, by using a sterile auger. Samples were collected in sterile bags and immediately placed on ice. Samples were appropriately stored prior to the enzyme and microbial analyses detailed below.

2.2. Determination of physical and chemical properties of soil

Physico chemical properties of soils, including texture, bulk density, pH (H_2O), cation exchange capacity (CEC), total nitrogen (N), organic carbon (OC), extractable cations (calcium, magnesium, sodium, and potassium) and available phosphorus (Bray 1) were analyzed using standard methods of the Non-Affiliated Soil Analysis Work Committee (1990). Soils were ground and passed through a 2 mm sieve before analyses. Briefly, pH was determined from a 1:2.5 soil-water suspension using a pre-calibrated pH meter (pH 700, Eutech Instruments Pte Ltd, Singapore). The particle size distribution was determined by the Bouyoucos method. Cations and exchangeable cations were determined from soil ammonium acetate (1 M, pH 7) extracts by using Inductively coupled plasma – optical emission spectrometry (ICP-OES). Bulk density (BD) was determined by using a bulk density sampler core of known volume after overnight drying at 105°C.

2.3. Determination of beta-glucosidase and urease activities in soil

Determination of beta-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and urease (urea amidohydrolase, EC 3.5.1.5) activities were performed by the methods of Dick *et al*. (1996) and Kandeler and Gerber (1988). The analyses were performed on topsoil samples only because previous studies have indicated that soil enzymes activities are mostly concentrated in

the topsoil (0–15 cm) region (Das and Varma 2010). Soil samples were passed through a 2-mm sieve and oven dried at 40° C prior to beta-glucosidase and urease activity assays of beta-glucosidase and urease activities.

2.4. Microbial community analyses

2.4.1. Extraction of total community DNA

Total community DNA was extracted from soil by using ZR Soil Microbe DNA extraction kit (Zymo Research, Irvine, CA, USA) according to the instruction of the manufacturer. DNA integrity and concentration were determined by agarose gel electrophoresis and fluorometric quantification (Qubit 2.0 fluorimeter, Invitrogen, California, USA), respectively, prior to downstream analyses.

2.4.2. PCR-Denaturing gradient gel electrophoresis (PCR-DGGE) analyses of soil microbial communities

PCR-DGGE analysis was performed to obtain a snapshot of the microbial (bacterial and fungal) community in soils. Universal primers 341F (5′-CCTACGGAGGCAGCAG-3′) and 907R (5′ CCG TCAATTCCTTTGAGTTT-3′) were used for amplification of the bacterial 16S rRNA gene, while primers ITS3 (5′-GCATCGAT GAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990) were used for amplification of fungal internally transcribed spacer-2 (ITS2) region. A 40 bp GC clamp was attached to the 5′-end of all forward primers (Muyzer *et al.* 1993). PCR was performed in a C100^{TM} thermal cycler (Bio-Rad Laboratories, CA, USA). Each PCR reaction contained 12.5 µl OneTaq 2X master mix (New England Biolabs, MA, USA), 0.2 µM of each primer, 15 ng DNA template and PCR-grade water to a final reaction volume of 25 µl. PCR conditions for both bacterial 16S rRNA and fungal ITS2 amplifications were exactly as described by Ezeokoli *et al.* (2016b). Thereafter, PCR products of all samples collected at similar depths from each site during a given season were pooled in equal proportion (per volume basis).

For DGGE, 30 µl of pooled PCR amplicons that included 6x loading dye at 3:1 mixture ratio, were loaded onto a 1 mm thick 6% (for bacteria) or 8% (for fungi) (w/v) polyacrylamide gel of denaturing gradient 40%-60% urea and formamide (100% denaturant is defined as 7 M urea and 40% (v/v) formamide). DGGE was performed on the Dcode™ Universal Mutation Detector System (Bio-Rad, Hercules, CA, USA) as described by Roopnarain *et al.* (2017). Following DGGE runs, gels were stained in 0.1% (v/v) ethidium bromide solution (in 1 x TAE buffer) and subsequently photographed using the ChemiDoc^{TM} MP imaging system (Bio-Rad Laboratories, USA).

2.4.3. Diversity analyses of PCR-DGGE fingerprints

DGGE gel images were subjected to densitometric analyses by using the Gene Tools software version 4.03.1.0 (Syngene, Cambridge, UK) as previously described by Mashiane *et al.* (2017). An unweighted (absence or presence) and weighted (presence and relative intensity of individual peaks calculated separately for each gel image) similarity matrix generated from band positions was subjected to hierarchical clustering using the average linkage method (UPGMA) in R software version 3.4.0 (R Core Team 2013). Furthermore, diversity indices, including Shannon-Weiner diversity index (*H′*) and species evenness (*J′*), were estimated based on the assumption that different species (sequence) migrate to different positions in the DGGE gel (Muyzer and Smalla 1998). *H′* and *J′* were computed in the vegan package of R software.

2.4.4. Sequencing, operational taxonomic units (OTUs) clustering and taxonomic assignments of dominant PCR-DGGE bands

Dominant bands on the DGGE gels were excised with a sterile scalpel and eluted of DNA as described previously by Ezeokoli *et al.* (2016b). Precisely two microlitres of the eluted DNA were used as the template for a PCR 're-amplification' involving the same set of primers stated earlier, but without the 40 bp GC-rich clamp attached to the forward primers. PCR amplicons were further purified and sequenced (Sanger sequencing) at the Central Analytical Facilities of Stellenbosch University, Cape Town, South Africa. Sequence electropherograms were manually inspected and edited for ambiguous nucleotides by using Chromas Lite (v.2.1, Technelysium Pty Ltd, South Brisbane, Australia). Edited sequences were clustered into OTUs at 97% 16S rRNA gene (for bacteria) and ITS2 sequence (for fungi) similarities as described by Ezeokoli *et al.* (2016a). OTU representatives were assigned into taxonomic ranks through the Ezbiocloud (https://www.ezbiocloud.net/) and UNITE (https://unite.ut.ee/analysis.php) databases for 16S rRNA gene and ITS2 sequences, respectively.

Sequence data have been deposited in the GenBank under the accession numbers KY985473-KY985518 (for bacterial) and MF001318-MF001351 (for fungi).

2.5. Statistical analyses

Soil physico-chemical data for each soil horizon were analyzed separately by the aligned rank transform of non-parametric factorial (site x season) analysis of variance (ANOVA) by using the ARTool package in R software version 3.4.0 (R Core Team 2013). Beta-glucosidase and urease activities data were subjected to a two-way (site x season) ANOVA with weighted means by using R software. Prior to ANOVA, enzyme activity data were square root-transformed to near-normality and to meet homoscedastic assumption for parametric tests. After ANOVA, the Tukey honest significant difference (HSD) post hoc test was used to separate significant means at $P < 0.05$. Pearson correlation was used to test the relationship between enzyme activities (normalized by square root transformation) and soil physico-chemical properties by using the R software. Based on the outcome (i.e., significant differences, $P < 0.05$) of ANOVA, correlations were conducted across sites, as well as on a site-by-site basis. Furthermore, to test the relationship of the microbial communities with the physico-chemical properties at each sampling depth, a redundancy analysis (RDA) was performed in the vegan package (Oksanen *et al.* 2015) of R software by using the unweighted similarity data matrix generated above (Section 2.3.4) and soil physicochemical properties. Thereafter, the environmental factors (vectors) were then fitted into the RDA model and their significance tested by permutations using the 'envfit' function of the vegan package.

3. Results

3.1. Physico-chemical properties of soils

Soils (topsoil and subsoil) were characterized as predominantly sandy loam. However, soils from mine B contained higher clay contents compared to other mines and reference soils (Supplementary Table S1 and Table S2). pH was generally acidic (pH 4.3–5.8) (Table S1 and Table S2). BD was highest in mine C (1.76 ± 0.22 g cm^{-3}) and ranged between 1.34 and 1.62 g cm^{-3} in the reference site and between 1.22 and 1.80 g cm^{-3} in soil stockpiles across seasons (Table S1 and Table S2). CEC was between 3.19 and 8.52 cmol kg^{-1} 100 g^{-1} for reference soils and between 2.39 and 10.94 cmol kg^{-1} in soil stockpiles. OC ranged from 0.46 to 1.71% in reference soils, and from 0.50 to 4.40% in soil stockpiles. In both topsoils and subsoils, total N of the reference soil was lower than in soil stockpiles of mine B and mine C (Table S1 and Table S2). Consequently, the C: N ratio was lower in most stockpiles (particularly mine B and mine C) compared to the reference site.

3.2. Beta-glucosidase and urease activities in soils

Two-way ANOVA revealed that site and the interaction between factors (site and season) had significant effects ($P < 0.05$) on beta-glucosidase and urease activities (Fig. 1). In winter, the differences observed in the beta-glucosidase between mine C and mine A and between reference site and mine A are significant (factors interaction effects, Tukey HSD $P < 0.05$). By contrast, only the differences in urease activity between the winter values of the reference site and summer values of mine B was significant (interaction effects, Tukey HSD $P < 0.05$). There were more significant differences (based on factors interaction) in beta-glucosidase activity amongst treatments (Fig. 1A) than in urease activity (Fig. 1B), suggesting that beta-glucosidases may be more sensitive to environmental factors compared to ureases. Overall, with the exception of the spring season for all sites, beta-glucosidase activity in stockpile soils was mostly higher than those of the reference soils (Fig. 1A). This suggests a higher biological activity in the stockpiled soils in response to availability of higher (compared to unmined soils) organic carbon in most of the soil stockpiles (See Table S1). Similarly, among sites, the urease activity was mostly higher (except in the spring season) in stockpile soils than in unmined soils (Fig. 1A). Across sites, beta-glucosidase activity was only positively correlated with C: N in mine A (Pearson's correlation coefficient, r = 0.91, $P = 0.013$) (data not shown), while urease activity was significantly correlated with the sum of exchangeable cations (S-V) only in the reference site (Pearson's correlation coefficient, r = 0.57, $P = 0.021$) (data not shown).

3.3. PCR-DGGE profile of microbial communities in soil across seasons

The PCR-DGGE profiles shown in Fig. 2 suggest that bacterial and fungal diversities of unmined soil are higher than those of stockpiles in the topsoil horizon (see also Supplementary Table S3 for some alpha diversity indices). In the topsoil, the least microbial diversity was observed in Mine A: with bacterial diversity lowest in the spring while fungal diversity was lowest in winter. Across seasons, the soil microbial diversity did not vary greatly within the unmined reference soil (Fig. 2 and Table S3), whereas varying levels of differences were observed in bacterial and fungal diversities amongst seasons in each of the coal mining sites (Table S3). Based on the associations depicted by the weighted hierarchical cluster dendrograms in Fig. 2, microbial community structure of the unmined reference soil is dissimilar to those of stockpiles in all three seasons. Within the reference site, summer and winter microbial community structures were more similar compared to the spring season in both topsoil and subsoil (Fig. 2B, Fig. 2D, Fig.

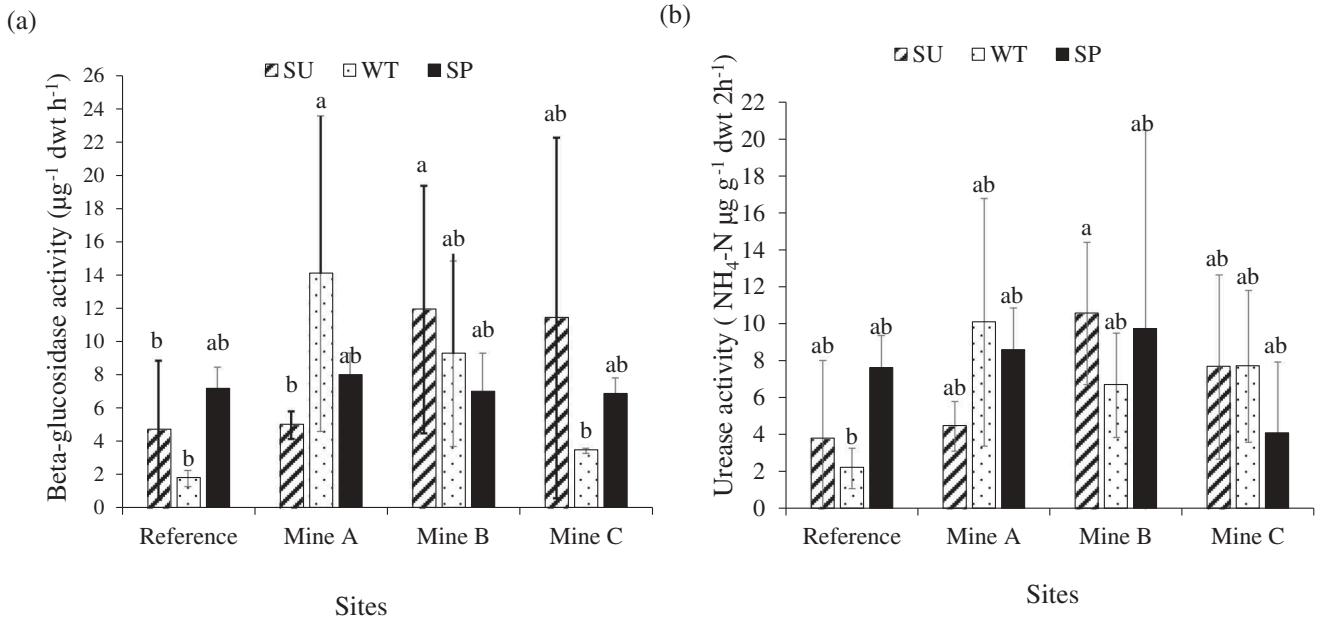

Figure 1. Mean enzymatic activities in soils (a) Beta-glucosidase activity (b) Urease activity. Sample size (N) ≥ 5. Bars with different letters are significantly different based on the effect of interactions between sites and season (Tukey HSD, $P < 0.05$). Error bars are standard deviations from means. SU, summer; WT, winter; SP, spring.

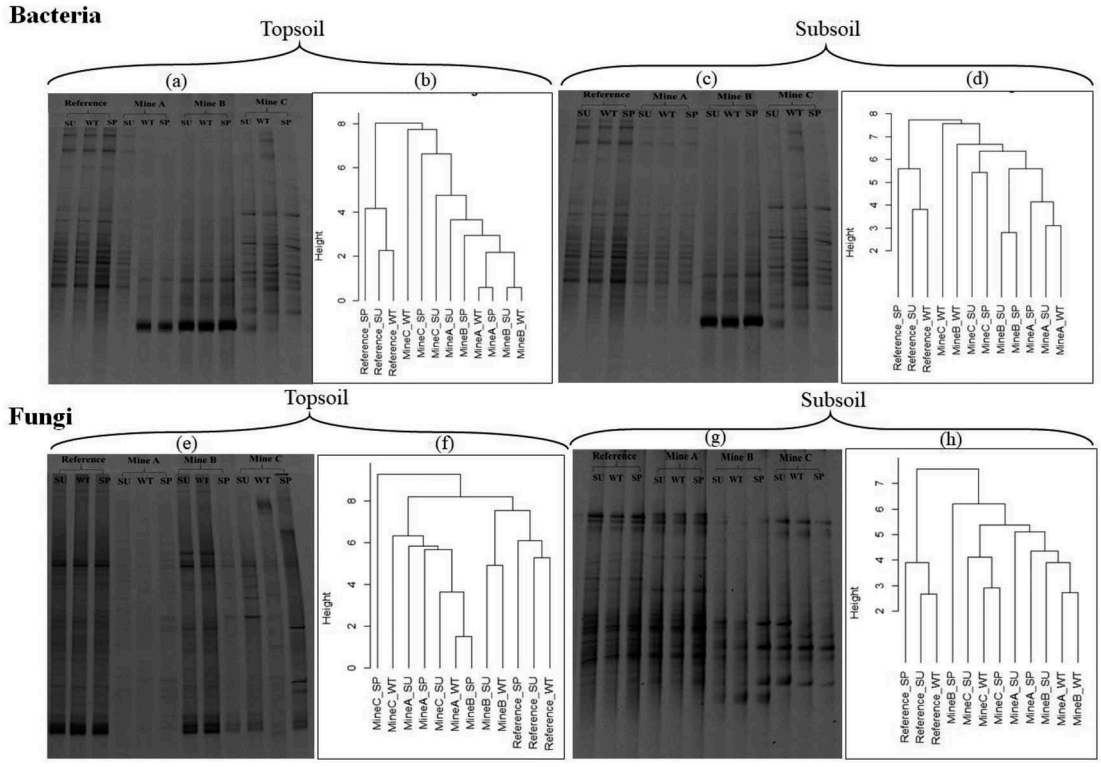

Figure 2. PCR-DGGE gel image and weighted hierarchical cluster dendrogram of microbial communities in soils. (a-b) Bacterial 16S rRNA gene diversity in topsoils (c-d) Bacterial 16S rRNA gene diversity in subsoils. (e-f) Fungal ITS2 gene diversity in topsoils. (g-h) Fungal ITS2 gene diversity in subsoils. SU, summer; WT, winter; SP, spring See also Supplementary Fig. S3 for weighted hierarchical cluster dendrogram.

2F and Fig. 2H). While microbial communities were mostly clustered based on site, close associations were observed in microbial community structure between some sites, such as the subsoil fungal community structures of mine A and mine B in winter (Fig. 2H).

3.4. Taxonomic affiliations of OTUs obtained from excised PCR-DGGE bands

A total of 44 bacterial and 33 fungal sequences were successfully (without ambiguous nucleotide base positions) obtained from the excised bands. These sequences yielded seven bacterial and 19 fungal OTUs (Table 1). Taxonomically, the bacterial OTUs spanned 2 phyla [Firmicutes (4 OTUs) and Proteobacteria (3 OTUs)] and four genera viz *Bacillus* (3 OTUs), *Pseudomonas* (2 OTUs), *Azomonas* (1 OTU) and *Lysinibacillus* (1 OTU) (Table 1 & Supplementary Fig. S1). All 19 fungal OTUs belonged to the Ascomycota phylum and included species of *Alternaria, Aureobasidium, Austroplaca, Cenangium, Claviceps, Curvularia, Dendroclathra, Diaporthe, Fusarium, Helotiales, Macrohilum, Neoscytalidium, Phialocephala, Phoma, Pyrrhospora, Thyronectra* and *Valdensinia* (Table 1 & Fig. S2).

3.6. Relationship between soil physico-chemical properties and microbial communities

The RDA model for the species-environmental relationship for topsoil as depicted in the biplot of Fig. 3A is not significant ($P = 0.725$), suggesting that physicochemical properties in the topsoil did not influence the microbial community of the topsoil in general (Table S4). In contrast, the RDA model for the species-environmental constraints in the subsoil is significant ($P = 0.011$). Specifically, in the subsoil, pH, organic carbon, total nitrogen and phosphorus contents of the subsoil significantly ($P < 0.05$) influenced the microbial community of the subsoil (Table S4). In the fitted model (correlations of environmental variables with both axis of the RDA model), the correlation of the first two axes with the silt content is significant ($R^2 = 0.566$, $P = 0.027$) (Table S5), suggesting that the silt content is a predictor of topsoil microbial community. In the subsoil, total nitrogen ($R^2 = 0.544$, $P = 0.039$) and phosphorus ($R^2 = 0.559$; $P = 0.030$) were the predictors of the soil microbial community structure (Table S5).

Based on the RDA biplot of Fig. 3, the microbial community of the topsoil and subsoil in the reference and mine C sites are least influenced by physicochemical parameters. However, in mine A and mine B, selected physico-chemical properties influence seasonal variations in the soil microbial communities. For example, pH, and cation exchange capacity of the topsoil strongly influenced the microbial community of mine A in summer (Fig. 3A), while in winter and spring, phosphorus and total nitrogen content of soil influenced microbial communities of mine A. The biggest predictor of the microbial communities in mine B subsoils during winter and spring is phosphorus (Fig. 3B).

4. Discussion

With respect to the hypothesis of this study, the pattern of differences observed in the physico-chemical properties (except for the total exchangeable cations in the topsoil

Table 1. Taxonomic affiliations (closest relative) and economic importance of operational taxonomic units obtained from sequenced excised DGGE bands.

	Ezbiocloud match		
†OTU no.	‡Closest relative	% Similarity	Potential role in agroecosystems (Reference)
Bacteria			
1	*Bacillus gaemokensis*	100	Unknown
2	*Pseudomonas paralactis*	100	Unknown
3	*Bacillus zhangzhouensis*	100	Unknown
4	*Bacillus amyloliquefaciens*	100	Non-pathogenic, plant-growth promoter (Gül *et al*. 2008; El-Daim *et al*. 2014)
5	*Azomonas macrocytogenes*	100	Nitrogen fixation (Page and Collinson 1987)
6	*Lysinibacillus macroides*	100	Potential bio-control agent, plant-growth promoter (Xiang *et al*. 2017)
7	*Pseudomonas matsuisoli*	98.6	Unknown
Fungi			
1	*Aureobasidium pullulans*	99.8	Endophyte, bio-control agent of blue mold in Rocha pear (Ferreira-Pinto *et al*. 2006)
2	*Phoma herbarum*	99.4	Endophyte, herbicide (Neumann and Boland 2002; Vikrant *et al*. 2006)
3	*Fusarium fujikuroi*	99.4	Plant pathogen; *Bakanae* disease of rice (Carter *et al*. 2008)
4	*Claviceps purpurea*	99.4	Ubiquitous pathogen of cereals and grasses (Tudzynski and Scheffer 2004)
5	*Alternaria tenuissima*	96.9	Plant pathogen: leaf spot of *Aloe barbadensis* (Vakalounakis *et al*. 2016)
6	*Austroplaca soropelta*	99.4	Unknown
7	*Pyrrhospora arandensis*	99.2	Unknown
8	*Alternaria petroselini*	97.6	Plant pathogen: leaf blight of fennel (Bassimba *et al*. 2012)
9	*Curvularia trifolii*	99.4	Plant pathogen: leaf spot in Berseem clover (Khadka 2016)
10	*Neoscytalidium dimidiatum*	99.0	Plant pathogen: etiological agent of cancer, shoot blight and fruit rot of almond (Nouri *et al*. 2018)
11	*Dendroclathra lignicola*	99.4	Unknown
12	*Helotiales* sp.	99.4	Symbiotic root endophyte (Zijlstra *et al*. 2005)
13	*Macrohilum eucalypti*	98.3	Endophyte: phylloplane of *Eucalyptus* (Swart 1988)
14	*Phialocephala humicola*	99.3	Endophyte: promote growth of tomato seedlings (Mahmoud and Narisawa 2013)
15	*Valdensinia heterodoxa*	97.2	Plant pathogen: etiological agent of leaf blight in highbush blueberry (Nekoduka *et al*. 2012)
16	*Phoma* sp.	99.4	Endophyte. Plant pathogen: leaf spot on *Schisandra chinensis* (Strobel *et al*. 2011; Choi *et al*. 2014)
17	*Diaporthe foeniculina*	99.4	Plant pathogen: stem and shoot cankers on sweet chestnut (Annesi *et al*. 2016)
18	*Cenangium acuum*	99.4	Plant saprophyte: Plant litter decomposition (Millar 1974)
19	*Sordariomycetes sp*.	99.2	Endophyte: mineralization of nutrients (Khan *et al*. 2017)

†Operational taxonomic units at 97% 16S rRNA gene or ITS2 sequence similarity.
‡Closest relative denotes sequence with the best match in the GenBank. The identities of OTUs to species taxa level are not definitive since only partial marker gene sequences are used.

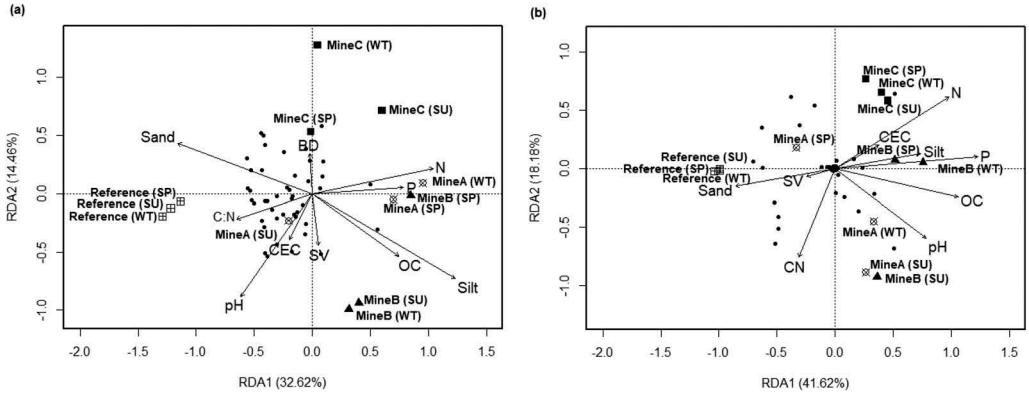

Figure 3. Redundancy analysis (RDA) biplot showing the relationship between soil physico-chemical properties and unweighted PCR-DGGE profile of microbial communities in soils. (a) Topsoil. (b) Subsoil. In both RDA plots, black dots are indicative of 'microbial species' in the context of combined (per soil horizon) number of different bands in the bacterial and fungal PCR-DGGE image of Fig. 2. Wherever present in site names, 'SU', 'SP' and 'WT' denotes summer, spring and winter samples. SV, sum of the exchangeable cations (Ca, Mg, Na, and K). See also Table S4 and S5 for the significance of the environmental factors in the RDA model.

horizon) of soils between unmined/reference site and stockpiles did not clearly suggest that stockpiled soils were in less adequate physico-chemical conditions. However, the differences appeared to be site-specific and variable across seasons (Table S1 and Table S2). Ghose (2004) reported drastic alterations in quality of topsoil dumps generated during opencast coal mining and that such alteration in soil quality increased with duration of storage. The high bulk density in some soil stockpiles soils may indicate high soil compaction attributable to heavy machineries, such as those used during the stripping and stockpiling process (Shrestha *et al*. 2005). Within a post-mining revegetation context, such high bulk density soils may pose restrictions on the growth of deep-rooted plants (Ghose 2004; Shrestha *et al*. 2005; Mensah *et al*. 2015). Higher organic carbon in coal mine stockpiles compared to the reference site may be due to the deposition of fossil (coal) dusts on the soil stockpile surfaces (Čížková *et al*. 2018). The observed acidic pH in all soils may be due to leaching of basic cations, as well as from the oxidation of sulfide minerals and acid mine drainage (Katzur and Haubold-Rosar 1996; Pietrzykowski 2014).

The observed higher beta-glucosidase activity in stockpile compared to unmined soils is similar to the findings of

Claassens *et al*. (2012) who observed that the beta-glucosidase activity in coal discard and asbestos sites had a higher maximum value compared to reference sites. Similar observations of higher urease activities in stockpile soils may be attributed to the relatively higher total nitrogen present in most stockpile soils (Table S1). Generally, urease activity correlates with soil nitrogen, especially when a source of urea is available (Meyer *et al*. 2015).

According to Das and Varma (2010), enzyme activity in soil ecosystems is mostly dependent on organic carbon levels, soil type, soil composition and microbial community diversity and function. For example, numerous authors have reported that beta-glucosidase activity is sensitive to soil pH and management practices (Tabatabai and Dick 2002; Makoi and Ndakidemi 2008; Meyer *et al*. 2015). In contrast, Meyer *et al*. (2015) observed that beta-glucosidase and urease activities correlated significantly with soil carbon, NO_3^- and pH but that the correlations between beta-glucosidase and soil carbon, NO_3^- and pH were weaker compared to correlations with urease. Furthermore, standard deviations from the mean beta-glucosidase and urease activities were higher in soil stockpiles than in reference soils, suggesting higher intra-site variations within coal mines. Similarly, Claassens *et al*. (2012) observed higher variations in enzyme activities on disturbed sites (coal discard sites) compared to undisturbed reference sites. The higher variation may be attributable to the mixtures of soil horizons (physically observed on-site) and intra-site variations in ages of soil stockpiles which was not considered in the design of this study (due to of lack of comprehensive information on stockpile ages).

The observed highest number of different bands ('species richness') in the topsoil and subsoil of reference soils compared to stockpile soils at all three sampling seasons (Table S3), suggests soil disturbances during stockpiling impair microbial diversity. Similar observations in the microbial diversity of disturbed and undisturbed sites have been previously reported (Fresquez and Aldon 1984; Harris *et al*. 1989, 1993). The variation of microbial communities in response to environmental conditions is well documented (Habekost *et al*. 2008; Baldrian *et al*. 2010; López-Mondéjar *et al*. 2015). For example, López-Mondéjar *et al*. (2015) reported that the spring and summer bacterial community of temperate deciduous soils significantly differed from those of the autumn and winter seasons. The changes in microbial diversity across seasons may be due to different supply of nutrients (resource availability) as a consequence of certain seasonal (environmental) factors such as temperature and moisture (Rasche *et al*. 2011; Fekete *et al*. 2012). In the soil ecosystem, these factors influence the allocation of photosynthates to the soil by roots of primary producers, the inputs of fresh litter, and above- or below-ground biomass production (López-Mondéjar *et al*. 2015). Invariably, the alteration in plant-derived exudates and biomass influences microbial community structure and composition of the soil and may give rise to niche partitioning of microorganisms.

The biodiversity of soil microbial communities is vital to the sustainability of soil ecosystem functioning. The detection of specific phylotypes at only certain depths suggests a vertical niche differentiation of species in the soil stockpiles (Hansel *et al*. 2008). Generally, vertical and seasonal variations in nutrient and physical parameters predispose the selection (selective pressure) of species with different adaptability (e.g., adaptation to energy and nutrient limiting conditions) and capabilities (e.g., nutrient mineralization) along the soil physico-chemical differentiation profile (Hansel *et al*. 2008; Rasche *et al*. 2011; López-Mondéjar *et al*. 2015; Stone *et al*. 2015). Niche differentiation of species in the soil ecosystem is important for the sustainability of soil health through biological processes that regulate, amongst others, nutrient cycling (Lennon *et al*. 2012).

Although the partial 16S rRNA gene sequences used for taxonomic identification of bacterial OTUs may not be sufficient to delineate sequences into the species taxonomic rank, reference to the closest relative at the species level is used herein to facilitate discussions towards potential roles of these species in agroecosystems and for comparison with the scientific literature. The observation of dominant phylotypes of the Firmicutes and Proteobacteria phyla agrees with observations made in previous soil microbial ecology studies (Lennon *et al*. 2012; López-Mondéjar *et al*. 2015). The dominance of *Bacillus* spp. in these soils suggest that these species are well-adapted to the prevailing nutrient-limiting conditions of the soils and are likely contributors to ecosystem processes in these soils. Phylogenetically similar species of *Bacillus* detected in this study (taxonomic clade) have been associated with mining soils (Jamal *et al*. 2016; Oladipo *et al*. 2018). *Bacillus* spp. are functionally diverse and play roles in the mineralization of plant-derived materials, plant-growth promotion, humus formation, biocontrol of plant pests and the degradation of hydrocarbons in the soil (Garbeva *et al*. 2003; Mandic-Mulec and Prosser 2011). For example, strains of *Bacillus amyloliquefaciens* have been shown to promote the yield of tomato (Gül *et al*. 2008) and improve the tolerance of wheat to heat stress (El-Daim *et al*. 2014). Other phylotypes whose strains are known to have ecological relevance in the agroecosystem include *Lysinibacillus* (e.g., *L. macroides*) and *Azomonas* (e.g., *A. macrocytogenes*) (Table 1). Similarly, some *Pseudomonas* spp. are able to demineralize coal (Singh *et al*. 2016), degrade polychlorinated biphenyl and polycyclic aromatic hydrocarbons (Nam *et al*. 2014; Bello-Akinosho *et al*. 2016), perform soil ecological functions (Naseby and Lynch 1999), promote plant-growth (Wicaksono *et al*. 2017) and participate in the remediation of metal-contaminated soils (Wasi *et al*. 2013; Oladipo *et al*. 2018).

Interestingly, most of the fungal species detected in the present study are known pathogens of plants (Table 1). The presence of these pathogens in the soil suggests that these soils may also serve as reservoirs for economically important plant pathogens. Hence, within the context of post-mining land use, especially for agriculture, the use of these soils may have implications on the productivity of certain crops if adequate disease management practices are not implemented. In contrast, other fungal strains such as *Aureobasidium pullulans, Phialocephala humicola, Phoma herbarum* and *Sordariomycetes* sp. detected in this study have been reported to have beneficial importance to industry and agriculture (Neumann and Boland 2002; Ferreira-Pinto *et al*. 2006; Mahmoud and Narisawa 2013) (Table 1).

5. Conclusion

While there were no clear differences between the physico-chemical properties of soil stockpiles and unmined sites, the differences in enzyme activities (mostly beta-glucosidase) and microbial diversity between soil stockpiles and unmined sites suggests that biological components of the soil are quite sensitive to soil management practice and/or disturbance. The impaired (compared to an unmined site) microbial community structure and diversity observed in stockpiles may have negative implications for soil biological processes driven by microbes, especially those that are critical for nutrient cycling and ecosystem sustainability. More importantly, the alteration observed in microbial communities of soil stockpiles in comparison to the reference site may impair post-mining land use capability. In addition, several microbial species detected in both reference and stockpile soils are closely related to characterized phytobeneficial species. Thus, these soils may serve as sources for the bioprospection of microbes of both agricultural and industrial applications. Further studies are required to investigate the influence of storage durations on the properties and biological parameters of stockpile soils.

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