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Microbial biomarkers as an indicator of ecosystem recovery following surface mine reclamation

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Abstract

Increased disturbance of terrestrial ecosystems in recent years for purposes of mineral extraction has created interest in development and optimization of reclamation methodologies for these lands. Currently, criteria for judging surface mine reclamation success, or progress toward reclamation goals, predominantly rely on aboveground indicators that fail to account for the abundance and composition of soil microbiota, an essential aspect of soil health. To test the utility of fatty acid methyl ester (FAME) biomarkers as indicators of reclamation progress, FAME bacterial, fungal, and total biomass biomarkers extracted from soil of surface mine reclamation sites of different ages and an adjacent undisturbed site were compared with other indicators of reclamation progress and ecosystem stability. Our results indicate that FAME microbial biomarkers and soil organic matter (SOM) contents were greatly impacted by disturbance. Discriminant analysis of FAME bacterial, fungal and total microbial biomass biomarkers, although clearly able to discriminate between disturbed and undisturbed ecosystems, indicated a trend towards the undisturbed condition with reclamation age. The ratio of FAME bacterial to fungal biomarkers reflected changes in other indicators of soil health (SOM, inorganic N concentration), suggesting that this ratio is a useful indicator of reclamation progress.

Keywords: Surface mine reclamation; Soil quality indicators; FAME analysis; Bacteria/fungi ratio

1. Introduction

Surface mining results in severe disturbance of large land areas in the US and throughout the world. Criteria for judging reclamation success of these disturbed lands largely encompass only visually distinguishable aboveground indicators, such as soil erosion and vegetation coverage and diversity, and fail to account for the health or composition of the soil microbiota, which are the basis of all terrestrial ecosystems. This may be an important shortcoming considering the role these organisms play in soil formation, plant establishment, and transformation of soil organic matter, the basis of all terrestrial ecosystems. Disturbance of soil ecosystems that disrupts normal functioning, or alters the composition, of soil microbial communities is potentially detrimental to both short- and long-term ecological stability.

Soil microorganisms are very sensitive to environmental change (Turco et al., 1994), and significant degradation of the microbial community can occur following drastic disturbance, both in terms of total biomass and species composition (Harris et al., 1989, 1991, 1993; Insam and Domsch, 1988; Stahl et al.,

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1988; Visser et al., 1983). Measures of the fate of the microbial community following the initiation of reclamation efforts would therefore, serve as an indicator of restoration progress (Harris et al., 1991) and may give insights into potential ways to accelerate a restoration.

Studies of microbial responses to, and recovery from, drastic disturbance, such as that associated with surface mining, are few, partly due to the difficulties associated with microbial community analysis. The few studies conducted have generally used measures that provide little or no structural information (e.g. microbial biomass C). A rapid, reliable method for analysis of soil microbial communities is needed to provide restoration ecologists with information about the status of the microbial community. FAME analysis has shown promise for characterization of soil microbial communities (e.g. Buyer and Drinkwater, 1997; Buyer et al., 1999; Pinkhart et al., 2002). FAME analysis is advantageous in that it is not dependent upon microorganism culturability and provides relative biomass measures for total bacteria and fungi (Zelles, 1999; Pinkhart et al., 2002), a potentially useful indicator of ecosystem self-reliance and soil health (Bardgett and McAlister, 1999; Klein and Paschke, 2000). In the present study, we test the utility of FAME analysis for determining the relative success, or progress, of a surface mine reclamation and compare this data with above- and belowground indicators of reclamation progress.

2. Methods

2.1. Study site and sample strategies

Research was conducted at the Pathfinder Uranium Mine in the Shirley Basin of southeastern Wyoming. The landscape is a semi-arid, short-grass steppe with mean annual precipitation of 28 cm and persistent desiccating winds. Average annual temperature is 3.5 °C, with the lowest average minimum temperatures occurring in January (−16 °C) and the highest average maximum temperatures in July (26.3 °C). Undisturbed soils in the area are classified as Ustic Haplargids.

Following standard practice on surface mining sites, the topsoil was stripped and stockpiled until mining operations were completed, in this case for 10 or more years. Stored soil was then spread on top of overburden to a depth of 20–30 cm. Two sites, seeded with native and non-native grass, forb and shrub species in 1982 and 1996, were chosen on the basis of similarity of slope and aspect as well as proximity to one another (<50 m). Both sites were dominated by *Agropyron smithii* and, although this species was not seeded on either site, *Agropyron crestatum*. An adjacent undisturbed site, dominated by *Artemisia tridentata* Nutt. sp. *wyomingensis* (Wyoming big sagebrush) with an understory of small grass (predominantly *Poa* sp.) and forb species, was examined for comparison.

Soil samples (approximately 500 g) were collected from the 5 to 10 cm depth beneath the nearest dominant plant species and the nearest bare soil area not having plant cover within 20 cm along 100 m transects at the 0, 25, 50, 75, and 100 m points, for a total of 10 samples from each site. Subsamples for FAME analysis were stored at −20 °C prior to analysis. Subsamples for all other analyses were stored at 4 °C prior to analysis (<2 weeks).

2.2. FAME analysis

Frozen soil samples were lyophilized and 1 g was placed in 50 ml glass centrifuge tube with 10 ml 0.2 M KOH in methanol. Centrifuge tubes were then mixed and placed in a 37 °C water bath for 1 h with occasional shaking. One millilitre 1 N acetic acid and 5 ml hexane were then added, followed by mixing and centrifugation at 2000 rpm for 10 min. The hexane layer was removed and extraction repeated twice more with 5 ml hexane. Hexane layers were combined, evaporated under nitrogen until dry, dissolved in 100 μl of 1:1 hexane:methyl *t*-butyl ether, and transferred to GC vials. Analysis was performed using a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector as described by Buyer et al. (1999). Fatty acids were identified by retention time according to the MIDI eukaryotic method (Microbial ID, Inc. Newark, Del.). Fatty acid biomarkers used for total biomass, fungi and eubacteria are listed in Table 1.

2.3. Inorganic nitrogen, pH, EC, and organic matter

Inorganic N contents were determined by extracting 10 g soil samples with 1 M KCl. Suspensions were

Table 1
Fatty acid biomarkers used for estimating relative total microbial biomass, fungi, and bacteria

Group	Fatty acids[a]
Biomass	14:0
Fungi	18:2 ω6c
Eubacteria	15:0, 17:0 cyclo, 19:0 cyclo, 15:1 iso, 17:1 iso, 17:1 anteiso

[a] See Cavigelli et al. (1995), Frostegård et al. (1993), Zelles et al. (1994), Zelles et al. (1995) and Zeller et al. (2001).

filtered through Whatman no. 42 filter paper and NH_4^+ and NO_3^- concentrations determined colorimetrically using a Technicon 2 inorganic N analyzer. The pH and electrical conductivity (EC) were determined using the saturation paste pH and saturation paste extract EC methods of Gavlak et al. (1994). Soil organic matter was quantified as the amount of soil carbon oxidized during reaction with $Cr_2O_7^{2-}$ and sulfuric acid using the method of Mebius (1960).

2.4. Canopy-coverage and species diversity

Canopy cover was estimated using the Daubenmire canopy-coverage method (Daubenmire, 1959). A metal frame (30 cm × 60 cm) was placed at eight random locations within each site. The total number of species present was determined and percent coverage of grass, forb, and woody species visually estimated.

2.5. Data analyses

Analysis of variance was performed to determine differences between soil chemical and microbiological characteristics of reclaimed and undisturbed sites. Discriminant analysis was also used to assess the relative similarity of soil microbial communities in soil collected from under plant canopies in reclaimed and undisturbed ecosystems. Discriminant analysis is a multivariate statistical technique that can be used to classify subjects in categories based on a series of test variables, in this case FAME biomarkers for eubacteria, fungi, and total biomass. Distance between group centroids and the rate of correct classification for each site were used to evaluate the relative similarity of the microbial communities between sites. Relative distance between reclaimed and undisturbed sites should be indicative of the relative success of microbial community reestablishment, with short distances associated with more complete microbial community reestablishment. The rate of correct classification was calculated by self-crossing the database and is the percentage of cases that were actually classified by the analysis in the correct category. The ability of discriminant functions to discriminate between reclaimed and reference sites should indicate that restoration of the microbial community is not complete, if the goal is to restore these communities to a prior to disturbance state. Incorrect classification, on the other hand, suggests microbial community similarity and, hence, successful microbial community restoration. All statistical analyses were conducted using the SPSS for Windows software package (Version 10.0.7).

3. Results

The undisturbed site had significantly greater plant species diversity ($P < 0.001$), averaging 6.2 species/0.18 m^2, than the 1982 and 1996 reclamation

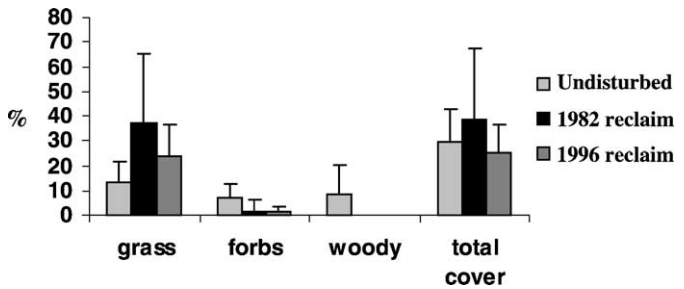

Fig. 1. Percent grass, forb, shrub, and total plant cover in undisturbed and reclaimed ecosystems as determined by the Daubenmire canopy-coverage method. Error bars show 1 S.D.

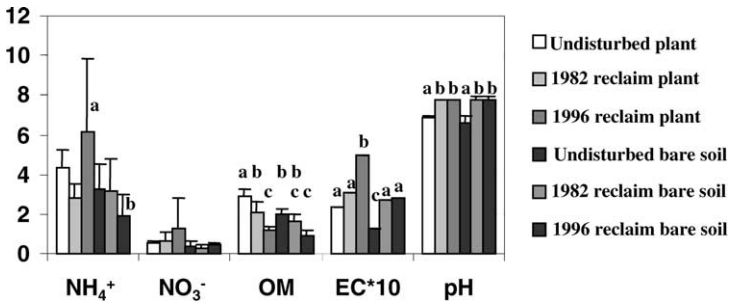

Fig. 2. Inorganic N concentrations (mg kg per soil), percent SOM (%), and values for EC (dS/m) and pH in reclaimed and undisturbed ecosystems. Different letters indicate significant differences at $\alpha = 0.05$ (one-way ANOVA, Bonferroni adjustment). Error bars show 1 S.D.

sites, which averaged 2 and 2.4 species/0.18 m^2, respectively. Forb and woody species cover was also significantly greater ($P < 0.001$) on the undisturbed site (Fig. 1), although no significant differences in total vegetation cover were found between any site.

Texturally, soil of the undisturbed site was found to be a sandy loam, whereas soils of both reclaimed sites are clay loams. Water content averaged 3.15 and 12.1% (w/w) for undisturbed and reclaimed soils, respectively.

Soil pH ranged from 6.63 for undisturbed bare soil to 7.8 for bare soil of the 1996 reclamation. The pH of both bare and plant-associated undisturbed soils was found to be significantly less than either reclamation site (Fig. 2). Soil EC was also found to be lowest in undisturbed soils, with highest values in 1996 reclamation and lowest in undisturbed bare soils (Fig. 2).

Soil NH_4^+ concentration ranged from 1.9 to 6.14 mg kg per soil, with both values found in 1996 reclamation bare soil and plant canopy associated soils, respectively (Fig. 2). Only the 1996 bare and plant-associated soil NH_4^+ concentrations were significantly different ($P < 0.05$). Although highest soil NO_3^- concentrations were found in 1996 plant-associated reclaimed soils, no significant differences were found between sites or between bare and plant-associated soils.

FAME total biomass markers for samples taken under plant canopies in the undisturbed and 1996 reclamation sites were significantly greater than in the 1982 reclamation plant-associated soil and all bare soil

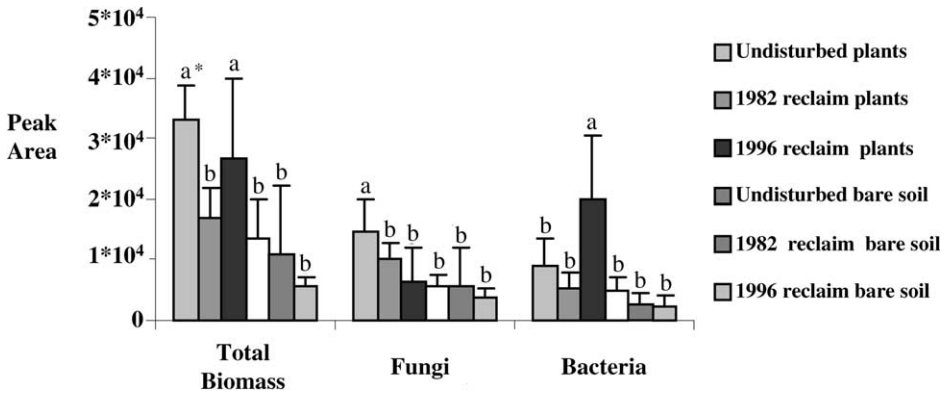

Fig. 3. FAME total biomass, fungal and bacterial biomarkers in reclamation and undisturbed soils. Different letters indicate significant differences at $\alpha = 0.05$ (one-way ANOVA, Bonferroni adjustment). Error bars show 1 S.D.

Table 2
Structure loadings and group centroids for discriminant analysis of microbial communities

	Structure loadings[a] function		Group centroids[b] function	
	One	Two	One	Two
Biomass	0.161	0.905	2.070	0.475
Fungal	0.306	0.159	−0.153	−1.411
Bacterial	−0.298	0.671	−3.572	0.556

[a] Pooled within-groups correlations between discriminating variables and canonical discriminant functions.
[b] Canonical discriminant functions evaluated at group means.

associated samples, while no significant differences were found between bare soil samples or the 1982 reclaimed plant-associated soil (Fig. 3).

Undisturbed plant-associated samples had significantly higher FAME fungal biomarker values than undisturbed bare-soil areas or either reclamation site (Fig. 3). No other significant differences between sites were found for fungal biomarkers.

Plant-associated 1996 reclaimed soil exhibited significantly greater values for FAME bacterial biomarkers (Fig. 3). No other significant differences were found for bacterial biomarkers. The ratio of bacterial to fungal FAME biomarkers for plant-associated soils were found to be 2.8, 0.5, and 0.6 for 1996 and 1982 reclamation sites, and the undisturbed site, respectively.

The ability of the discriminant functions to discriminate between plant-associated samples from all three sites on the basis of total biomass, fungal and bacterial FAME biomarkers was found to be significant (Wilks $\Lambda = 0.073$ and 0.542, $\chi^2(4) = 39.3$, $\chi^2(2) = 9.2$, $P < 0.001$ and $P = 0.01$ for functions one and two, respectively). Homogeneity was affirmed using Box's *M*. Structure loadings (Table 2) indicate that fungal and bacterial biomarkers are highly correlated with discriminant function one, while biomass and, to a lesser extent, bacterial biomarkers had the highest correlation with discriminant function two.

Classification yielded only a single misclassified sample, a sample from the undisturbed site that clustered with the 1982 reclamation site. Canonical scores indicate that the microbial community of the 1982 reclamation site is more similar to the undisturbed site than is the 1996 reclamation site (Fig. 4).

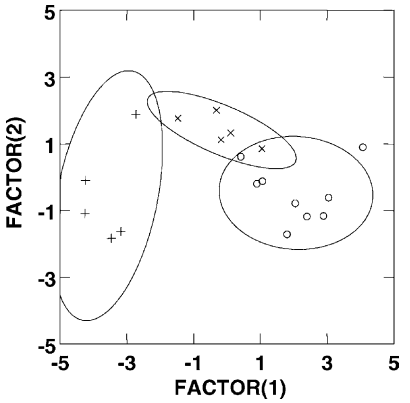

Fig. 4. Plot of canonical scores calculated by discriminant analysis for the identification of the undisturbed (reference) site (○), the 1982 reclamation site (×), and the 1996 reclamation site (+). Ninety percent confidence ellipses for the patterns of each site are also shown.

4. Discussion

Any strategy intended to evaluate reclamation success of disturbed lands requires criteria for judging restoration progress. These criteria should reflect ecosystem viability and long-term stability. Typically, threshold values for any indicator of restoration success are determined by comparison with nearby undisturbed sites that are thought biologically stable and representative of pre-disturbance conditions (White and Walker, 1997). Although use of the pre-disturbance condition as a benchmark for evaluation of reclamation success of drastically disturbed ecosystems is questionable, because many attributes of the pre-disturbance condition may be unattainable due to severe alteration of edaphic characteristics for example, nearby undisturbed ecosystems have developed sustainable structural and compositional characteristics under similar environmental controls. Therefore, the degree of similarity between reclamation sites and the pre-disturbance condition should provide evidence for establishment of conditions conducive to development of long-term ecosystem stability if indicators are carefully chosen and used with their limitations in mind (White and Walker, 1997).

Analysis of FAME biomarkers, for example, is unlikely to provide detailed information about the presence and abundance of many key species that may be critical to long-term stability largely because our

knowledge of soil microbial community structure and associated fatty acid profiles is very limited (Zelles, 1999; Pinkhart et al., 2002). In addition, although individual fatty acids, or fatty acid profiles, are thought to be specific for groups or individual species of bacteria and fungi, the concentration and composition of each fatty acid is affected by nutritional status and other environmental factors (Pinkhart et al., 2002). However, while FAME analysis is not able to quantify all key microorganisms in the soil environment, a number of studies have demonstrated the utility of fatty acids for determining relative changes in the abundance of broad groupings of microorganisms, such as bacteria and fungi (White et al., 1979; Bardgett and McAlister, 1999; Zeller et al., 2001; Bailey et al., 2002). Similarities between relative abundance of fungal, bacterial, and total microbial biomass of reclaimed and undisturbed reference ecosystem soils should indicate reestablishment of gross microbial community structure and, thus, biophysical conditions conducive to ecological stability (Zelles, 1999). Increased dissimilarity between relative microbial biomass fractions of reference and reclaimed ecosystems with reclamation age, or decreased total biomarker amounts, is potentially indicative of progressive ecological degradation and restoration failure. Therefore, FAME analysis may be highly suitable for providing restoration ecologists with a measure of overall soil health and restoration progress.

Discriminant analysis of soil collected from under plant canopies was clearly able to discriminate between undisturbed and both reclamation sites on the basis of fungal, bacterial, and total biomass FAME biomarkers (Fig. 4 and Table 2). Moreover, classification analysis yielded only a single misclassified sample, an undisturbed site sample classified with the 1982 reclamation. However, plotting canonical scores shows lesser distance between the undisturbed and 1982 reclaimed ecosystems than the 1996 reclaimed ecosystem, indicating a trend with reclamation age towards the undisturbed state (Fig. 4). Fungal and bacterial biomarkers contributed the most to function 1 (Table 2), indicating that these variables were most important for differentiating between sites.

An important indicator of reestablishment of soil microbial community stability and, hence, ecosystem self-regulation, is the relative proportions of bacterial and fungal biomass (Bardgett and McAlister, 1999; Klein and Paschke, 2000; Zeller et al., 2001). Soil microbial communities of undisturbed terrestrial ecosystems tend to be dominated by fungal microbial biomass and pathways of decomposition (Bardgett and McAlister, 1999). Disturbance is known to be especially detrimental to fungal populations and a number of studies have shown that physical disturbance results in increased bacterial dominance (Beare et al., 1992; Guggenberger et al., 1999; Frey et al., 1999; Stahl et al., 1999). The ratio of the bacterial to fungal FAME biomarker in the 1996 plant-associated soil is about 2.8, while this ratio is very similar for 1982 reclaimed and undisturbed plant-associated soils (approximately 0.5 and 0.6, respectively). The high biomass of 1996 reclamation plant-associated soil is composed of predominantly bacteria, while the fungal component of this soil is similar to values for disturbed and undisturbed bare-soil areas (Fig. 3). Nineteen years after reclamation was initiated, total biomass biomarkers of the 1982 reclamation were significantly less than the plant-associated soils of both the undisturbed and 1996 reclamation sites, mostly due to decreased bacterial biomass, while fungal biomarkers exhibit a slight increase over that of the 1996 reclamation (Fig. 3). FAME analysis therefore, indicates that 1982 soils, while supporting lower overall microbial abundance than undisturbed soil, are approaching relative fungal and bacterial abundances similar to what was found in the undisturbed ecosystem.

In most natural ecosystems only small amounts of N are annually attained from N_2 fixation and atmospheric inputs, thus N must be retained and cycled efficiently in order to maintain ecosystem productivity. Disturbance can drastically alter the N cycle and many years may be required for disturbed lands to equilibrate to a state in which N inputs equal, or are greater than losses (Reeder, 1985). Typically, N uptake by plants and soil microorganisms of undisturbed ecosystems results in little inorganic N accumulation (DeLuca and Keeney, 1993). High soil inorganic N concentrations are potentially subject to greater losses from leaching, volatilization, or conversion to gaseous forms. The relative amount of soil inorganic N in reclaimed and reference ecosystems is therefore indicative of N cycling efficiency and, hence, ecosystem stability. Plant-associated soil of the 1996 reclamation had significantly greater NH_4^+ concentrations than

both 1982 reclaimed and undisturbed soils, and the highest NO_3^- concentration. While this indicates that N is available for plant growth, it also suggests N cycle inefficiency. Inorganic N content of the 1982 reclamation soil was not significantly different from that of undisturbed soil, suggesting reestablishment of a more efficient and tighter N cycle. These results suggest that changes in relative bacterial and fungal FAME biomarker abundance reflect changes in soil inorganic N concentrations and, hence, N cycle efficiency.

Typical of soil reclaimed after surface mining (Insam and Domsch, 1988; Harris et al., 1989), our results indicate that disturbance was highly detrimental to SOM pools (Fig. 2). Soil storage is known to be detrimental to SOM, primarily due to decreased plant organic matter inputs (Harris et al., 1989). The effects of soil mixing, first when soil was stripped and again spread prior to seeding, would also be expected to negatively impact SOM pools in a manner similar to tillage. Lower SOM in reclaimed soils may, however, be partially due to incorporation of potentially low OM subsurface horizons into the soil mix that was spread prior to seeding.

Although low SOM contents are generally less important to plant reestablishment than N and P availability for example, SOM is very important to sustained nutrient cycling and establishment and maintenance of soil physical characteristics. Continued loss of SOM after reclamation is therefore highly indicative of ongoing ecological degradation and restoration failure.

Soil organic matter content was significantly less for the plant-associated 1982 reclamation soil than undisturbed plant-associated soil but significantly greater than 1996 reclamation soils (Fig. 2), indicating partial recovery of SOM pools. Increased SOM with reclamation age provides evidence for reestablishment of the soils ability to retain and cycle nutrients. Differences between plant-associated and bare soil associated SOM content also increased with reclamation age (Fig. 2), underscoring the importance of plant cover to SOM reestablishment.

Two additional soil chemical characteristics that potentially influence restoration success are EC and pH. Salts migrating upward from overburden materials directly underneath topsoil is problematic for many surface mine reclamations (Jurinak et al., 1987). Similarly, the chemical nature of overburden materials can strongly influence soil pH over time (Tucker et al., 1987).

Electrical conductivity was low for all soils, although EC for the 1996 reclamation site was found to be significantly greater than for both the 1982 and undisturbed sites (Fig. 2). However, a relatively high correlation ($r^2 = 0.53$, $P = 0.04$) was found between soil NO_3^- concentration and EC values for reclaimed soils, suggesting that soil NO_3^- is responsible for much of the higher EC values found for the 1996 reclamation soil and that high salt concentration is unlikely to compromise the ecological integrity of these sites in the near future.

Both reclamation sites had significantly higher pH values than the undisturbed site (Fig. 2), although not to an extent likely to result in excessive nutrient limitations or toxicity problems (Tucker et al., 1987). Increased reclamation soil pH may be due to dilution of slightly acid topsoil with subsoil buffered to a higher pH, or movement of materials up from overburden.

A commonly used aboveground indicator that an ecosystem, as a whole, has recovered from disturbance is recovery of previously existing energy capture rate (Beedlow et al., 1988). The plant cover data (Fig. 1) suggests that energy capture rates in the 1982 reclamation site are equal to, or greater than, that of the undisturbed ecosystem. While similar energy capture rates for reclaimed and undisturbed sites suggest successful reclamation, disturbed site plant species richness was found to be greatly reduced. Although native shrub and forb species made up a large part of the seed mixture of both reclamation sites, no shrub species were found and most forbs were weedy species. Because greater plant species richness is generally associated with increased resource use efficiency, ecosystem stability and resilience (Myers, 1996), greatly reduced plant species richness suggests reclamation is not complete and may take many years to approach the undisturbed condition. Further research is needed to elucidate relationships between belowground indicators of plant cover and species richness and belowground indicators of ecosystem recovery.

Another aboveground indicator of ecosystem stability, and therefore, restoration success, is surface soil stability. Neither reclaimed site exhibited evidence of gross soil erosion, such as pedestals around plant bases, suggesting reclamation by this indicator was successful.

In conclusion, our study exemplifies the utility of FAME biomarker analysis as an indicator of surface mine restoration progress. Our results suggest that the relative amounts of FAME bacterial and fungal biomarkers may be a valuable indicator of surface mine reclamation progress, reflecting trends toward ecosystem stability demonstrated by other indicators of reclamation progress.

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