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SOIL MICROBIOLOGY

Soil Functional Diversity Analysis of a Bauxite-Mined Restoration Chronosequence

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Abstract Soil microorganisms are sensitive to environmental perturbations such that changes in microbial community structure and function can provide early signs of anthropogenic disturbances and even predict restoration success. We evaluated the bacterial functional diversity of un-mined and three chronosequence sites at various stages of rehabilitation (0, 10, and 20 years old) located in the Mocho Mountains of Jamaica. Samples were collected during the dry and wet seasons and analyzed for metal concentrations, microbial biomass carbon, bacterial numbers, and functional responses of soil microbiota using community-level physiological profile (CLPP) assays. Regardless of the season, un-mined soils consisted of higher microbial biomass and numbers than any of the rehabilitated sites. Additionally, the number and rate of substrates utilized and substrate evenness (the distribution of color development between the substrates) were significantly greater in the un-mined soils with carbohydrates being preferentially utilized than amino acids, polymers, carboxylic acids, and esters. To some extent, functional responses varied with the seasons but the least physiological activity was shown by the site rehabilitated in 1987 indicating long-term perturbation to this ecosystem. Small subunit ribosomal DNA (SSUrDNA)-denaturing gradient-gel electrophoresis analyses on the microbiota collected from the most preferred CLPP substrates followed by taxonomic analyses showed Proteobacteria, specifically the gamma-proteobacteria, as the most functionally active phyla, indicating a propensity of this phyla to out-compete other groups under the prevailing conditions. Additionally, multivariate statistical analyses, Shannon's diversity, and evenness indices, principal component analysis, biplot and un-weighted-pair-group method with arithmetic averages dendrograms further confirmed that un-mined sites were distinctly different from the rehabilitated soils.

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Introduction

A growing body of evidence suggests that changes in bacterial and fungal community structure and function can provide early signs of alternations in soil productivity and even predict restoration success. Restoration ecology has been extensively reviewed in several recently published articles (19 and references contained therein) with the consensus that further work is required to assess the role(s) of microorganisms in facilitating soil formation and reversing the detrimental effects of anthropogenic influences, enhancing biodiversity, and ecosystem function of perturbed environments.

Bauxite mining in Jamaica is performed by the opencast method and is likely a major threat to soil productivity by inducing perturbations to the soil microbiota that drive nutrient cycles. In the open cast bauxite mining method, the top 0–3 cm soils are stripped off and stock-piled alongside

the mined areas. Once mining is no longer economically feasible, rehabilitation of is attempted by reshaping the area using the stockpiled topsoil, and re-vegetating with grass and other crops. Environmental impacts of such surface mining operations are potentially long lasting because of the widespread destruction of vegetation prior to mining activities, surface runoff of organic matter, and the overall degradation of soil structure. Current mining regulations in Jamaica require bauxite industries to restore mined regions to the level of ecological integrity that may have existed prior to the mining activities. It has been shown previously that the rehabilitated mined soils are different than the undisturbed soils [22, 25, 27]. However, the complex physico-chemical and biological changes that occur as a function of mining activities, it is rather difficult to gauge the ecological effects and restoration success of mined regions.

Previously, we have reported the detrimental effects of environmental perturbations on microbiota from wetland soils and aquatic systems [4–6]. We hypothesized that bauxite mining activities likely result in long-lasting impacts to the microbial metabolic activities that support agricultural productivity and maintain soil health. In this context, a chronosequence provides for the opportunity to evaluate changes in soil quality over time [12], representing variable time series of soils undergoing ecological succession under similar conditions. This approach permits the assessment of ecosystem change over time without having to wait for the change(s) to occur, and has previously been used to assess soil degradation or improvement by comparing the soil physico-chemical and microbial properties in a restoration project in Florida's Everglades National Park [33]. In this study, we used a multi-pronged approach to obtain comparative analyses on the bacterial functional diversity in un-mined and rehabilitated bauxite soils of various ages (0, 10, and 20 years old) located in the Mocho Mountains of Jamaica. Our results indicate that the rehabilitation of bauxite-mined ecosystems are complex and influenced by a conglomeration of abiotic and biotic factors. Despite decades of rehabilitation attempts, it appears that mining activities severely impaired the ecosystem productivity of mined regions.

Methods

Soil Sampling

Samples for this study were obtained from the Mocho Mountains of Jamaica (18°03′33″N; 77°33′33″W; Fig. 1). This region is well known for rich bauxite deposits and has been actively mined in the past. Samples were collected from within three chronosequence sites and an adjacent site that has never been used for mining purposes (un-mined, site 32). The chronosequence sites are a decade apart in restoration stages such that site CC24 was rehabilitated in 1987, site F25 in 1997 and more recently, site C9 in 2007. The un-mined site is freely grazed upon by animals such as goats and cows and consisted mainly of grasses with interspersed mango (*Mangifera indica*) and tamarind (*Tamarindus indicus*) trees. The plant species that grow in the rehabilitated sites mainly consisted of the African Star grass (*Cynodon nlemfuensis* Vandercyst), but sparse populations of ferns and tropical shrubs such as black sage (*Cordia curassavica)* and bamboo (*Bambusa vulgaris*) were also recorded from site CC24 (1987). Similarly, in site F25 (1997) and C9 (2007), we also observed patches of Guinea grass (*Panicum maximum* Jacq.) and *Brachiaria* species.

The first sets of samples were collected in September, 2007, two weeks after the passage of Hurricane Dean. The second samples were collected in January 2008 during the dry season. These two months typically represent the peak of wet and dry seasons in Jamaica [31]. Soil samples were collected with an auger measuring 15 cm×7.5 cm. The aboveground plant and detritus layer were excluded. Randomly, 6–10 cores were collected from each sampling site, as shown in Fig. 1. Sampling locations were approximately 15–30 m apart and cores were collected at a depth of 10–15 cm. A total of 36 cores were collected for each sampling period. The auger was rinsed with 70% ethanol and air dried to prevent sample carry-over between sites. The samples were stored in 1.6 L Whirlpak® bags over ice and transported to the laboratory at Tallahassee, Florida within 72 hr of collection. Upon receipt, the samples were passed through a 2 mm sieve to remove coarse roots and debris and immediately processed for further analyses. Subsamples for DNA based analysis were stored at −70°C.

Biogeochemical Analyses

For biogeochemical analyses, each cored sample was analyzed separately. Soil moisture was calculated by drying approximately 20 g of soils in a forced-air drying oven at 70°C until constant weight. Metal concentrations were determined by digestion of dried, ground soil subsamples with nitric acid followed by inductively coupled argon plasma spectrometry (model Vista MPX CCD simultaneous ICP-OES manufactured by Varian, Inc., Walnut Creek, CA; Method 200.7, USEPA, 1993). Microbial biomass carbon was determined by the methods of fumigation-extraction [36]. Triplicate samples were extracted with 20 ml of 0.5 M K_2SO_4 and filtered through #42 Whatman filter paper. Dissolved organic C was determined on a Shimadzu total organic carbon analyzer. Microbial biomass carbon was determined by subtracting the extractable total organic carbon in the triplicate controls (non-fumigated) from the triplicate chloroform-treated samples [37].

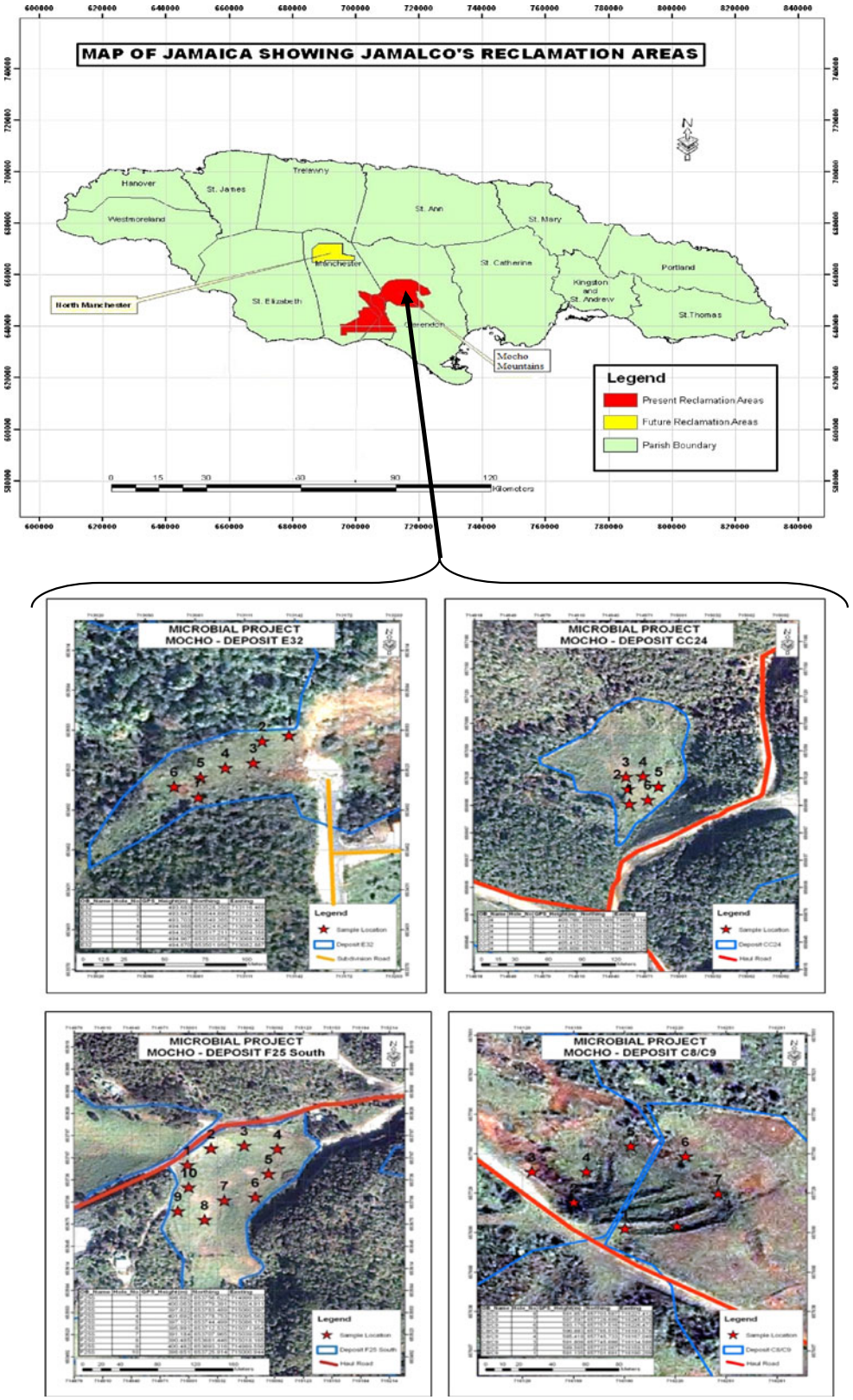

Figure 1 Shown are sampling site locations in the Mocho Mountain area of Jamaica. Studies were conducted with soils taken from the un-mined site (18°6′28″ N, 77°18′28″ W) and sites that were rehabilitated in 1987 (18°4′45″ N, 77°19′58″ W), 1997 (18°5′46″ N, 77°20′ 42″ W), and 2007 (18°5′32″ N, 77°20′47″ W). *Stars* represent GIS locations where replicate soil cores were collected at the un-mined and rehabilitated sites

Bacterial Analyses

Most Probable Number Analysis

Most probable number (MPN) values were obtained by homogenizing soil cores and pooling the samples to represent one sample each from the un-mined site, 1987 site, 1997 site, and 2007 site, respectively. Bacterial abundances in the soil samples were estimated by a three-tube MPN technique, as previously described [5], with minor modifications. Briefly, 10 g of soil was added to 90 ml of 0.85% NaCl and stirred for 30 min. Soil debris was allowed to settle for 10 min and 0.3 ml of supernatant was serially diluted in microtiter plates that contained Luria-Bertani medium. Control tubes consisted of un-inoculated medium and incubated at approximately 25°C for a week. Growth was compared between samples obtained from rehabilitated and un-mined soils and bacterial numbers were calculated using EPA software version 4.0 [23].

Community-Level Physiological Profiles

Community-level physiological profiles (CLPPs) of un-mined and chronosequence samples were analyzed using Biolog® Ecoplates as shown previously [14, 21]. The Biolog® Ecoplate consists of different substrates that are widely found in soils [20, 22]. Similar to the serial dilutions prepared for MPNs, soils from un-mined and chronosequence sites were diluted and 150 μL from the 10^{-3} dilutions were inoculated into Biolog EcoPlates (Biolog Inc., Hayward, CA). This way, each of the 96-well plates contained three replicates of each sample exposed to 31 different carbon sources. Water blanks were included in each plate and incubated at 25°C without shaking. Substrate utilization was monitored by measuring absorbance at 595 nm using a BIOTEK UQuant Microplate Spectrophotometer, (BIO-TEK Instruments Inc., USA). The first measurement was made immediately after inoculation and subsequent readings were taken after at 24-h intervals for 7 days. The measurements of individual substrates were corrected for background absorbance by subtracting the absorbance of the control (water) samples. If a negative number was obtained, it was manually set to zero. A well was considered positive if the mean OD_{595nm} exceeded that of 0.25, as previously reported [13]. Additionally, average well-color development (AWCD) was also calculated by averaging the final absorbance values for all substrate wells, as shown previously [13]. Using the AWCD values of 31 carbon sources for each sample prior to statistical analysis eliminates variable responses that may occur as a function of differences in initial cell densities [7, 13, 14].

DNA Extraction from CLPP Substrates and PCR Amplification

Functional diversity of bacteria in the most preferred substrates after 7 days of incubation were studied by amplification of ribosomal genes and separation by denaturing gradient-gel electrophoresis (DGGE). CLPP substrates can be grouped into five-category carbohydrates, carboxy acids, amino acids, esters, and polymers. From each category, we collected 100 μL of biomass from the most preferred substrates; DNA was extracted from the centrifuged pellets using the UltraClean™ Microbial DNA Isolation Kit (MoBio, Inc.) and estimated by the ND-1000 Nanodrop spectrophotometer (Starlab, USA). Bacterial 16S rDNA from the CLPP samples were amplified using universal eubacterial primers GM5 forward (5′-GC-clamp-CCTACGGGAGGCAGCAG-3′) and 907 reverse (5′-CCGTCAATTC(A/C)TTTGAGTTT-3′) spanning the region V3 of the 16S rRNA gene [29]. The polymerase chain reaction (PCR) was prepared using ready-to-go PCR beads (GE Healthcare), 2 μL (approximately 15 ng) template DNA, PCR grade sterile water, and 0.4 pmol/μL of each primer. Amplification was performed in a thermocycler (Biorad, Hercules, CA) using denaturation at 95°C for 3 min, followed by 35 cycles of a minute each at 95°C, 55°C, and 72°C, followed by a 10 min final extension at 72°C. Product size and purity were confirmed by electrophoresis in 1% agarose gels stained using 1x SybrSafe (Invitrogen, USA).

Denaturing Gradient-Gel Electrophoresis

DGGE was performed using the Dcode™ Universal Mutation Detection System (Biorad, Hercules, CA). The polyacrylamide concentration in the gel was 6.5%, and the gels were made with a linear denaturing gradient ranging from 35% denaturant at the top of the gel to 60% denaturant at the bottom. The 100% denaturant solution contained 7 M urea, 40% (*v*/*v*) formamide, 6.5% (*w*/*v*%) acrylamide/bis-acrylamide (37.5:1), and 1×TAE buffer (40 mmol L^{-1} Tris, pH 8.3; 20 mmol L^{-1} sodium acetate; 1 mmol L^{-1} EDTA). A 1-cm gel without denaturant was cast on the bottom of the gradient gel as a sealant. The gels were allowed to polymerize for 2 h and then PCR amplicons (20 μL) were mixed with 10 μL of loading dye buffer (70% glycerol, 0.05% Bromophenol Blue, 0.05% Xylene Cyanole, 2 mM EDTA) and resolved in a 6.5% (*w*/*v*) polyacrylamide gel in 1.0×TAE buffer. Electrophoresis was carried out at 100 V for 16 h at 60°C. After electrophoresis, the gel was stained with SYBR Green I (1:10,000 dilution; Molecular Probes, Eugene, OR) for 30 min, viewed using a UV transilluminator (302 nm), and analyzed with the Quantity one analytical software (Biorad, Hercules, CA). Band analysis was performed by setting a background subtraction

at 15 using the rolling disk method. DGGE bands that consisted of intensity lower than 10% were excluded from further analysis. Banding patterns were then used to estimate statistical differences between the un-mined and chronosequence soils using diversity indices, species richness, and evenness.

DGGE bands of interest were then excised from the gel and soaked in 40 μL of MilliQ water overnight at 4°C. Five microliters of the supernatant was used for reamplification with the original primer set without the GC clamp. PCR products were purified with the QIAquick PCR-Purification Kit (Qiagen, Maryland, USA) and checked for purity in a 1% agarose gel. DNA concentration was quantified using a NanoDrop Spectrophotometer prior to sequencing.

DNA Sequencing and Phylogenetic Analysis

Purified DGGE bands were sequenced at the DNA Sequencing Laboratory at Florida State University using the forward primer. Sequences generated from this study were compared with sequences contained in the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using basic local alignment search tool, sequences from this study and affiliated sequences from the database were aligned and evolutionary relationships were inferred using the maximum parsimony method by MEGA4 [35]. Branches that resulted in bootstrap values of less than 50% have been shown as collapsed. The positions that contained gaps or data that was missing were eliminated.

Statistical Analyses

Biogeochemical analyses were performed in triplicate; mean values are reported in this study. Differences in physical and chemical properties between sites were tested by multivariate analyses using ANOVA and Tukey's tests ($P<0.05$) to estimate for pair-wise multiple comparisons between all the treatment means.

The 72-h data were used for statistical analysis of CLPPs [18]. BIOLOG substrate utilization patterns were analyzed to determine functional diversity [13] including substrate richness (the number of substrates utilized), substrate evenness (the distribution of color development between the substrates), and diversity as measured by the Shannon's diversity index ($H=-\Sigma$Pi log Pi, where Pi=(OD reading of well I)/(sum of all wells)) based on the OD in the Ecoplates. The Shannon's evenness (E) of catabolic diversity was calculated as $E=H/\ln S$, where S (Shannon's richness) is the number of substrates used by the bacterial communities. AWCD, diversity indices and evenness were compared using one-way ANOVA with GraphPad Prism 5.0 [26]. Principal component analysis (PCA) was used to characterize the functional structure of the bacterial community by classifying treatments according to their substrate utilization patterns and seasonal differences using MVSP 3.1 [24]. Differences in the physico-chemical and biogeochemical characteristics across soils were further analyzed using cluster analysis from the un-weighted pair-group method with arithmetic averages (UPGMA) to form linkage dendrograms. Additionally, UPGMA trees were generated for the DGGE banding patterns using Quantity One (Biorad, Hercules, CA) and assessed using the Dice coefficient of similarity index.

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences obtained in this study are available in GenBank under accession numbers of GQ853502–GQ853547.

Results

Biogeochemical Properties and Bacterial Enumerations

The soils were characterized as stony loam and rehabilitated soils contained relatively more limestone debris. Therefore, regardless of seasons, significantly elevated levels of calcium were observed as a function of chronosequence age (Table 1). Conversely, there was a significant decrease in the levels of cadmium with age of the rehabilitated sites. Additionally, using average values over the two seasons, we observed that the most recent chronosequence site (2007) had the lowest microbial biomass carbon. Bacterial numbers (MPNs) were approximately 2–4 logs higher in the un-mined soils compared with rehabilitated soils (Table 1). Surprisingly, in the dry season (January 2008), bacterial populations increased in all sites with the highest numbers observed from the un-mined soils (9.42×10^{12}). Conversely, lowest numbers were found in soils reclaimed in 1997 (1.42×10^{10}).

Additionally, principal components biplot analyses (Fig. 2) provided for comparative analyses of the sites according to physico-chemical and biological parameters. PCA biplots of environmental variables are indicated by arrows that point in the direction of increasing values of each variable and coordinates of the arrowheads indicate the degree of correlation with the axes. For the wet season, the first PCA axis explained 56.11% of variance and second axis explained 31.68% with a cumulative score of 87.79%. The primary axis (PC1) mainly represented the site elevation, iron (Fe), cadmium (Cd), and calcium (Ca) concentrations. The second axis (PC2) represented the sites by moisture, microbial biomass carbon, magnesium (Mg), and aluminum (Al) concentrations. However in the dry season, the first two axes (PC1 and PC2) explained a

Table 1 Comparative analyses of selected characteristics of un-mined and rehabilitated soils in the wet (September 2007) and dry (January 2008) seasons at the Mocho Mountain in Jamaica

	Un-mined	Reclaimed in 1987	Reclaimed in 1997	Reclaimed in 2007
Elevation (m)	494	410.4	397	590
Soil type	Stony loam	Stony loam	Stony loam	Stony loam
Wet season (September 2007)				
Moisture	20.41±0.82*	18.22±0.26	18.80±0.61	17.14±0.66*
Microbial biomass carbon (mg kg^{-1})	1,456±73.34*	545.70±11.24**	1,519±39.77*	895.80±21.85*
Mg (mg kg^{-1})	440.30±14.93	344.30±11.13	494.8±29.83	489.0±68.58
Ca (mg kg^{-1})	1,517±308*	6,664±1,697*	21,365±10,539**	25,068±9,304**
Al (mg kg^{-1})	168,050±2,657	175,620±4,367	164,680±5,695	163,559±3,472
Cd (mg kg^{-1})	45.13±5.28*	33.67±0.95*	28.70±1.36*	24.63±2.70**
Fe (mg kg^{-1})	98,829±1,307	98,196±1,025	94,905±3557	91,829±3,391
MPNs	7.4×10^{12}	4.6×10^{8}	4.6×10^{10}	1.1×10^{8}
Dry season (January 2008)				
Moisture (%)	18.79±0.44	17.89±0.16	18.54±0.45	14.61±0.73
Microbial biomass carbon (mg kg^{-1})	1,329±71.03*	1,645±86.98*	1,914±75.56**	1,013±56.29*
Mg (mg kg^{-1})	567.3±19.76	387.5±7.29	600.1±20.63	464.4±29.08
Ca (mg kg^{-1})	2,258±238.2*	5,118±380.0*	16,153±6,408**	19,826±6,708**
Al (mg kg^{-1})	195,667±3,644	196,255±3,309	189,912±4,227	187,783±4,101
Cd (mg kg^{-1})	54.96±5.46*	36.93±0.91*	34.61±2.53**	28.00±2.85***
Fe (mg kg^{-1})	110,445±2,285*	97,069±1,263**	104,894±1,559	99,978±2,360*
MPNs	9.4×10^{12}	1.9×10^{12}	1.4×10^{10}	1.8×10^{11}

Values given are±standard error. Sites with the same combination of lowercase letters indicate significant differences among sites ($P<0.05$). The absence of letters indicates no significant differences were found

MPN most probable number (values are expressed in units of per gram soils (wet weight) and are the average of three replicates)

* $P\leq0.01$, significantly different; ** $P<0.001$, significantly different; *** $P<0.0001$, significantly different

cumulative percentage of 78.15% of the variance (PC1 accounting for 52.76% and PC2 25.39%, respectively). The first axis PC1 was based on variations in moisture, Ca, Al, and Cd; whereas, the second axis PC2 was determined by elevation, microbial biomass carbon (MBC), Mg, and Fe.

Cluster analysis of the physico-chemical and biological parameters is also shown alongside in Fig. 2. Un-mined sites clustered separately from the rehabilitated sites with some degree of variability between seasons. In the wet season, all three rehabilitated sites (a) clustered separately to the un-mined site (b). When the same characteristics were analyzed for the dry season, again rehabilitated sites from chronosequence years 1997 and 2007 (a) clustered separately to the un-mined sites. However, rehabilitated site 1987 clustered with the un-mined site for the dry season, shown as group c in Fig. 2b.

Community-Level Physiological Profiling

The relative capacity for substrate utilization in the different soils is shown in Fig. 3. Significant differences ($P<0.0001$) were found in the AWCD between the un-mined soils collected during the wet season (September, 2007) and the sites reclaimed in 1987 and 2007 such that un-mined soils consistently showed higher AWCD. Conversely, no significant seasonal differences were observed between the un-mined and the 1997 reclaimed site. Additionally, there was a significant AWCD increase ($P<0.0001$) in site reclaimed in 1987 during the dry season. Statistical analyses on the 72-h AWCD values were performed because this was the shortest incubation time that allowed the best resolution between the sites. As shown in Table 2, both the bacterial diversity and evenness varied as a function of the chronosequence ages and seasons. The un-mined sites were consistently higher in both diversity and species evenness indices.

Furthermore, the 31 carbon sources in the CLPP assay can be grouped into five categories—eight carbohydrates, nine carboxylic acids, six amino acids, four polymers, and four miscellaneous compounds. The substrate utilization patterns from all soils are shown in Fig. 4. Carbohydrates were the most preferred substrates by the un-mined soil microbiota with 90% of the substrates being utilized in the wet season and 97% in the dry season. In the wet season, rehabilitated sites (1987 and 2007) showed significant differences ($P<0.001$) in the substrate utilization patterns when compared with the un-mined site and those that were

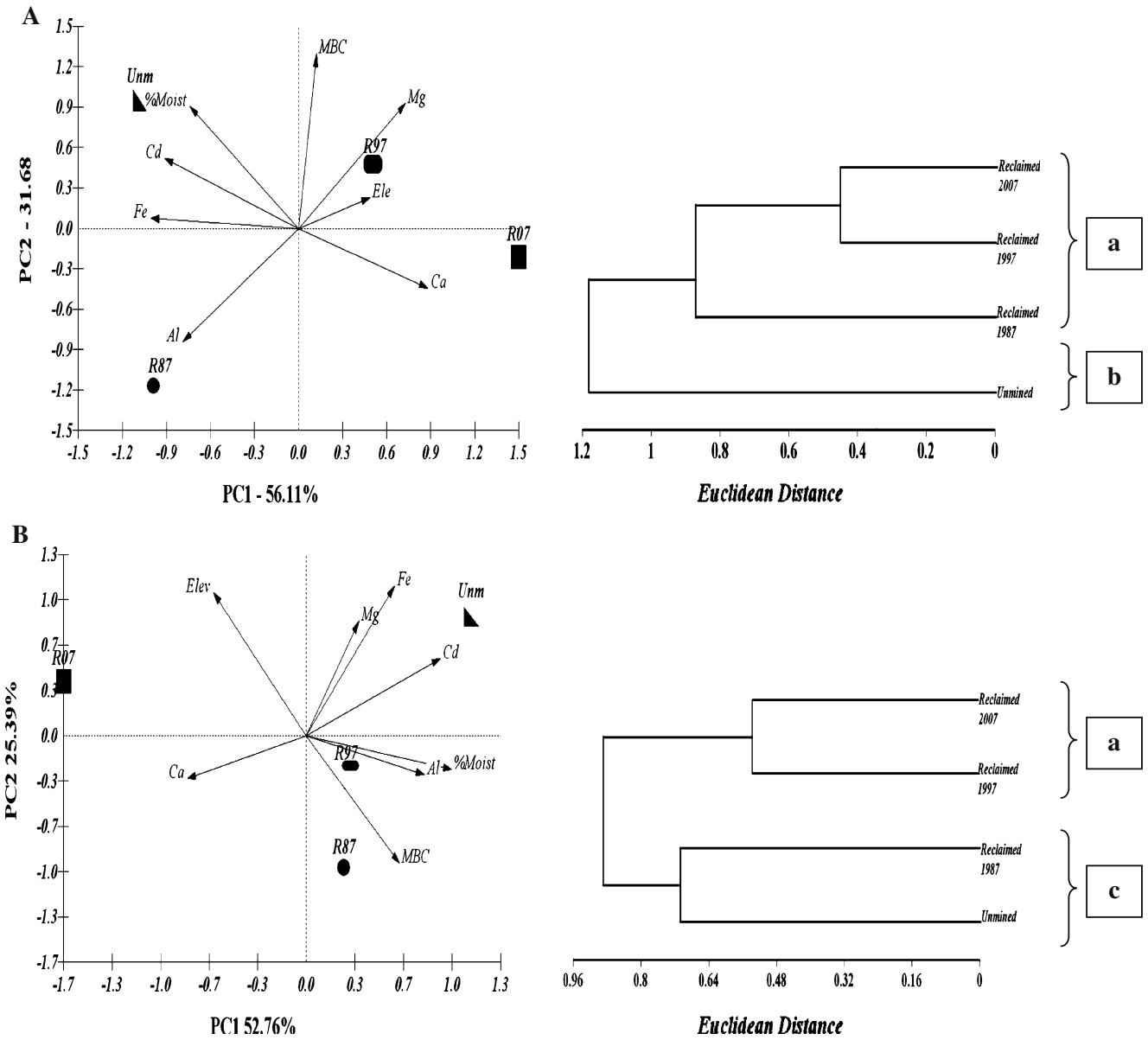

Figure 2 Principal component biplot analysis of soil physico-chemical parameters in the wet (**a**) and dry seasons (**b**). Also shown alongside are the cluster analyses showing groupings of sites. *Arrows* for the chemical and physical variables reflect the direction of increase for each variable, and the length of each *arrow* indicates the degree of correlation with the ordination axes

reclaimed in 1997. Surprisingly, in the wet season, only 6% of the combined substrates were utilized by the soils rehabilitated in 1987 and 16% by the soils rehabilitated in 2007 (Supplementary Fig. 1). Conversely in the dry season, a significant increase of approximately 81% was observed in the substrate utilization by the bacterial communities within the site 1987 along with an increase of 35% in the 2007 rehabilitated soils. No significant differences were observed in the substrate utilization patterns between the un-mined soils and those that were rehabilitated in 1997.

Additionally, PCA (Fig. 5a) and cluster analysis (Fig. 5b, c) were performed on the combined substrate utilization values from all sites and seasons. Similar to PCA and cluster analyses on the site characteristics, un-mined sites clustered together and separately from the restored sites with some degree of variability between seasons. Sites rehabilitated in 1997 were the closest to the un-mined sites in CLPP profiles, as shown by PCA and cluster analyses (Fig. 5). The first and second factors (PC1 and PC2) accounted for 57.97% and 14.29% of the variance with a cumulative variance sum of 69.26%, respectively.

Denaturing Gradient-Gel Electrophoresis

DGGE and cluster analysis of functionally active microbiota from the most preferred substrates are shown in Fig. 6. The

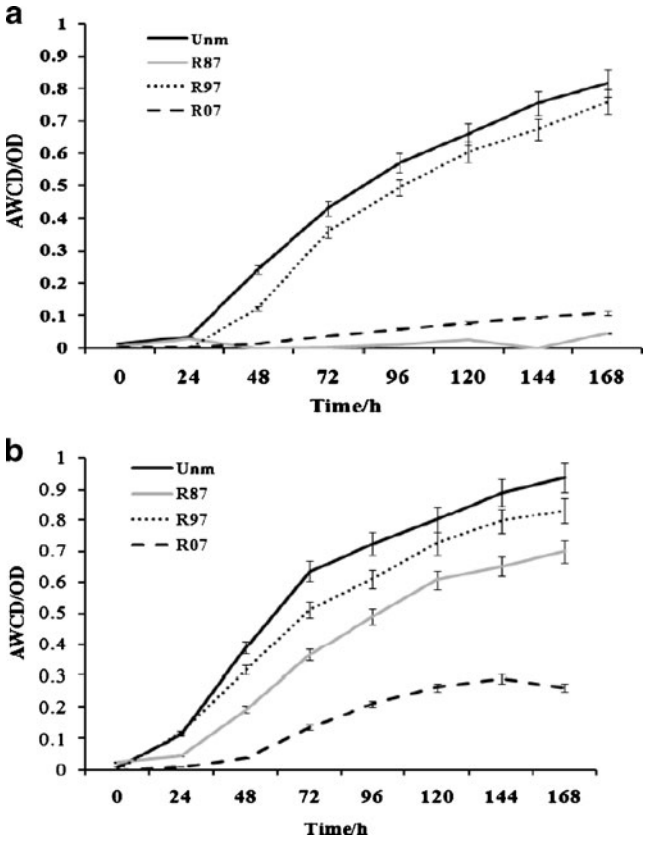

Figure 3 Average well-color development (*AWCD*) from the community-level physiological profile assay as a function of the metabolized substrates from the un-mined and rehabilitated soils collected from the Mocho Mountains of Jamaica. Shown are **a** AWCDs from the wet season and **b** AWCDs from the dry season

DGGE banding patterns were subjected to the Quantity One analytical software (Biorad, Hercules, CA) which indicated that un-mined soils consisted of approximately more than 250 different bands; far greater than the numbers identified from the rehabilitated sites which were approximately 65 (2007), 99 (1987), and 125 (1997), respectively (data not shown). As expected, the banding patterns varied depending on both the site and seasons. Moreover, UPGMA trees resulting from the banding patterns also clearly indicated the un-mined soils ranked higher on the Dice coefficient of similarity index, which is shown along the cluster analysis (Fig. 6). Conversely, chronosequence soils showed smaller indices, indicating distinct differences in the response of the microbiota to the CLPP substrates.

Taxonomic Affiliations

Taxonomic affiliations of microbiota identified from the actively metabolized CLPP substrates from the un-mined and restored soils are shown in Fig. 7 and Supplementary Fig. 2. Again, it was clear that the un-minded soils were far more diverse than any of the rehabilitated soils with some degree of variability observed along the seasons. Across all soils, two main phylogenetic divisions were predominant-*Proteobacteria* (95%) and *Bacteroidetes* (5%). Within *Proteobacteria*, the majority of the sequences belonged to γ-*Proteobacteria* (49%), and lesser numbers to β-*Proteobacteria* (30%), and α-*Proteobacteria* (16%). Among the bacterial species identified, the *Pseudomonads* were the most abundant group among the γ-proteobacteria while *Burkholderia* and *Rhizobium* were the most dominant ones belonging to α-proteobacteria and β-proteobacteria groups (Supplementary Table 1).

In the un-mined site and the 1997 rehabilitated site, there was generally a predominance of γ-proteobacteria from both the wet and the dry seasons. Conversely, β-proteobacteria predominated in the 2007 rehabilitated site and α-proteobacteria in the 1987 site, respectively.

Discussion

Bauxite mining activities potentially alters the soil productivity by affecting the physiological and functional attributes of microorganisms that remineralize nutrients and contribute toward soil productivity. Evidence from several studies indicates that both bacteria and mycorrhizae play critical role(s) in facilitating soil formation and recovery

Table 2 Shannon's diversity (*H*) and evenness (*E*)[a] for the soil microbial communities in the un-mined and rehabilitated soils in the wet (September 2007) and dry (January 2008) seasons at the Mocho Mountain in Jamaica

Site	Shannon's diversity index (*H*)	Shannon's Evenness (*E*)
Wet season		
Un-mined	3.09	0.9
Reclaimed 1987	1.426	0.686
Reclaimed 1997	2.999	0.944
Reclaimed 2007	2.17	0.666
Dry season		
Un-mined	3.223	0.938
Reclaimed 1987	2.774	0.808
Reclaimed 1997	3.08	0.897
Reclaimed 2007	2.749	0.801

[a] The 72-h average well-color development (AWCDs) from the CLPP assay were used to calculate the species diversity and evenness

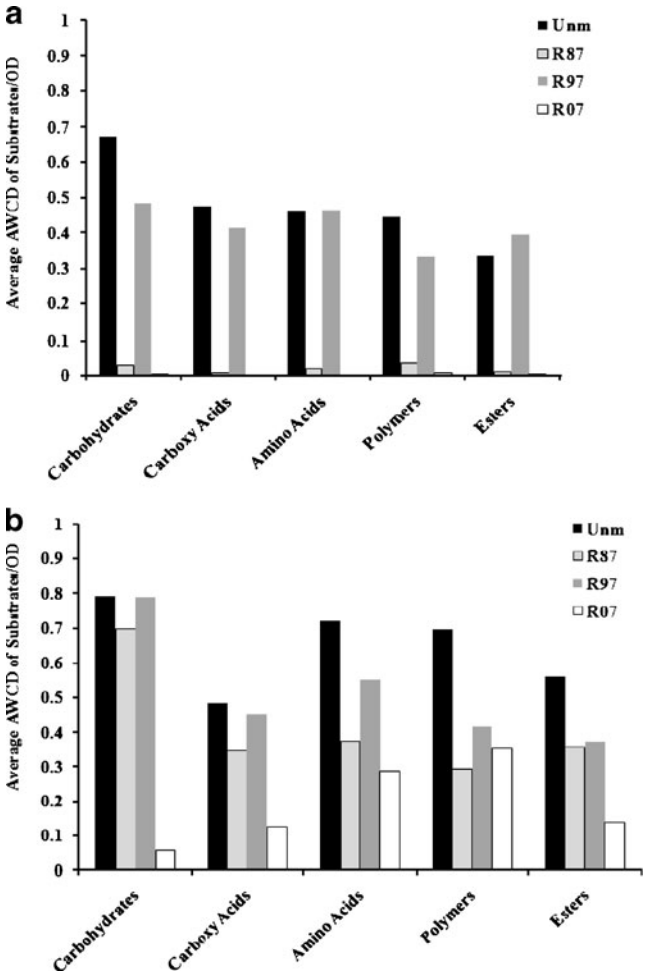

Figure 4 Utilization of carbon substrates in the community-level physiological profile assay by the soil microbial communities of the unmined and rehabilitated soils collected from the Mocho Mountains of Jamaica. Shown are **a** AWCDs from the wet season and **b** AWCDs from the dry season

processes of anthropogenically perturbed environments. Specifically, mycorrhizae hyphae intersperse the soil particles and soil bacteria secrete mucilaginous "glue" to form aggregates, setting the stage for establishment and eventual survival of pioneer plant communities (19 and references contained therein). Studies on rehabilitated Southwestern Australian soils have shown that it took 5 years for arbuscular mycorrhizal fungi to recover to pre-mining levels [22]. Similarly, even though ectomycorrhizal fungi were not well adapted to mining disturbances; they re-colonized by spore dispersal mechanisms and reached to pre-mining levels in 7 years. It was also shown that fungal species richness increased as a function of increasing time of rehabilitation in a previously mined site [11].

These studies have largely focused on fungal biota with less information available on the effects of mining activities on soil bacterial communities. To this end, we compared soil bacterial functional responses in samples collected from un-mined soils to those that were histroically mined but are in the process of rehabilitation for over two decades. Site CC24 was rehabilitated in 1987, site F25 in 1997, and more recently, site C9 began the process of restoration in 2007. Such rehabilitated soils provides an opportunity to study bacterial community shifts including immigration and emigration of soil microorganisms that play significant role (s) in recovery processes of such impacted environments. Our multi-pronged approach included physico-chemical measurements, MBC and microbial population estimates (MPNs), community-level physiological profiles (CLPPs)

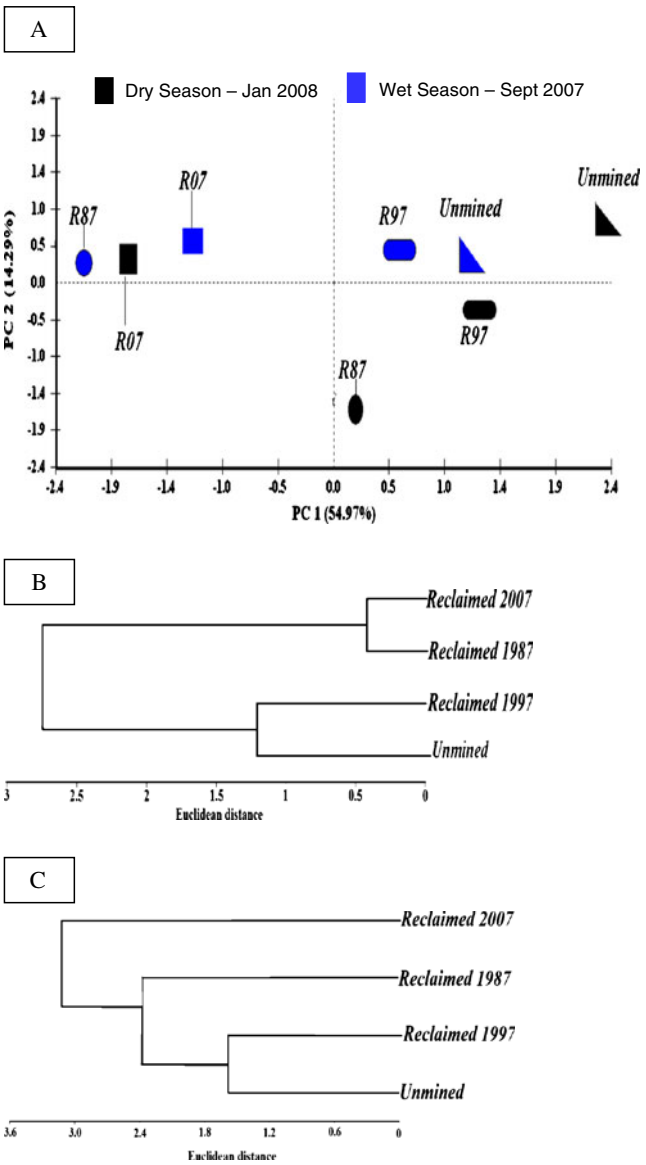

Figure 5 Principal component analysis of community-level physiological profile assay by the un-mined and rehabilitated soils collected from the Mocho Mountains of Jamaica. Shown are **a** PCA analyses; **b** and **c**, dendrogram analysis of microbial responses as a function of ecosystem status

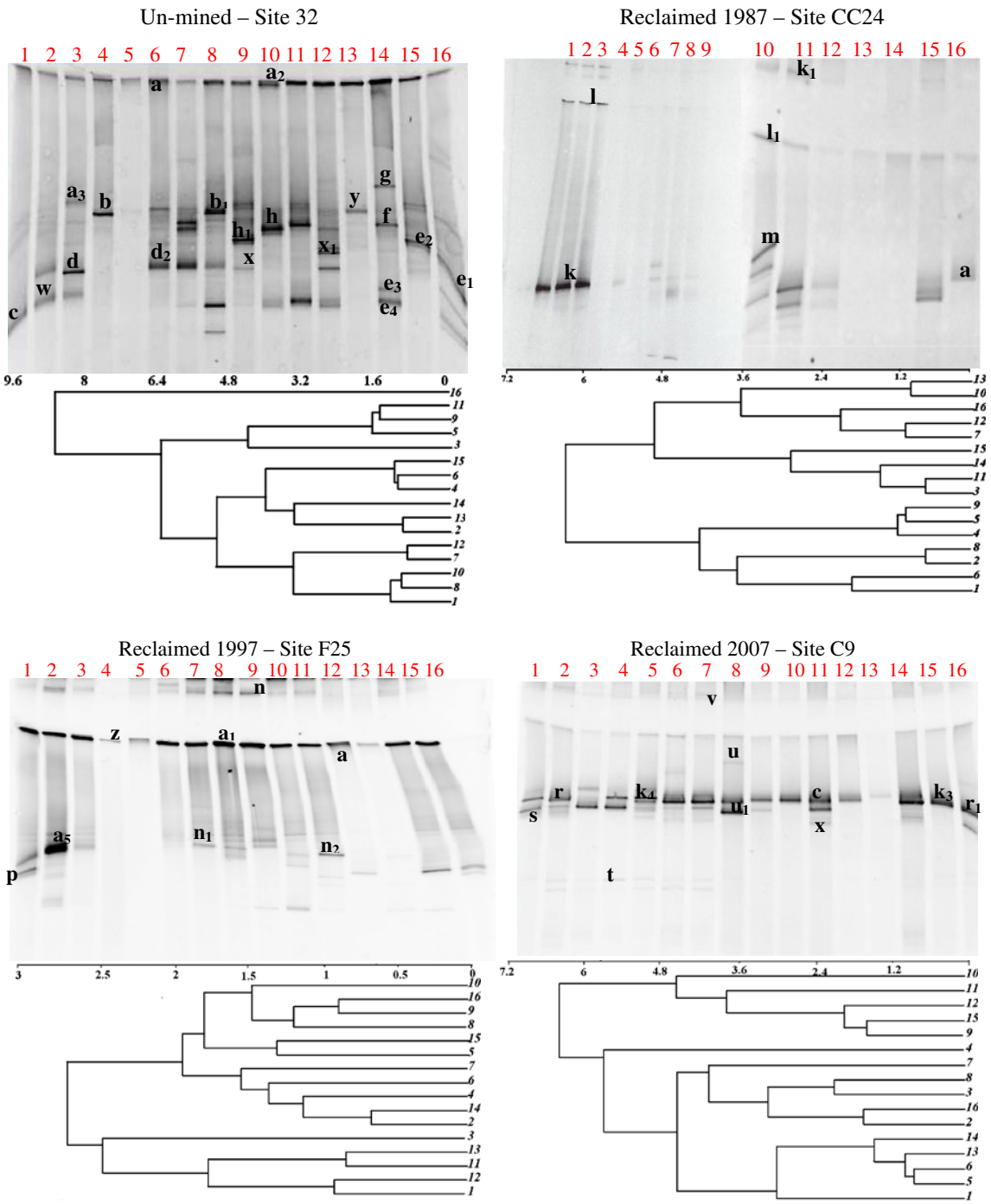

Figure 6 DGGE and dendrograms analyses of the eubacterial 16S rDNA from the most preferred substrates in the community-level physiological profile assay. Shown are substrates from wet season: *1* D-cellobiose, *2* *N*-acetyl-D-glucosamine, *3* D-glucosaminic acid, *4* 2-hydroxybenzoic acid, *5* L-asparagine, *6* L-serine, *7* Tween 40, *8* Tween 80, *9* pyruvic acid methyl ester. Substrates from dry season: *10* *N*-acetyl-D-glucosamine, *11* D-mannitol, *12* D-galactonic acid-lactone, *13* L-asparagine, *14* L-serine, *15* Tween 40, *16* Tween 80

and analyses of soil microbiota actively metabolizing the CLPP substrates. Multivariate statistical analyses, *H* and *E* indices, PCA, biplot, and dendrogram analyses, further confirmed that un-mined sites were distinctly different from the rehabilitated soils (Tables 1 and 2; Figs. 2, 3, 4, 5, 6, and 7).

Specifically, the un-mined soils were higher in MBC and bacterial numbers were approximately 2–4 logs higher than the rehabilitated soils (Table 2). Conversely, MBC in the 1997 rehabilitated soils were similar to the un-mined soils, indicating successful restoration of this particular site. Microbial biomass represents a significant portion of the

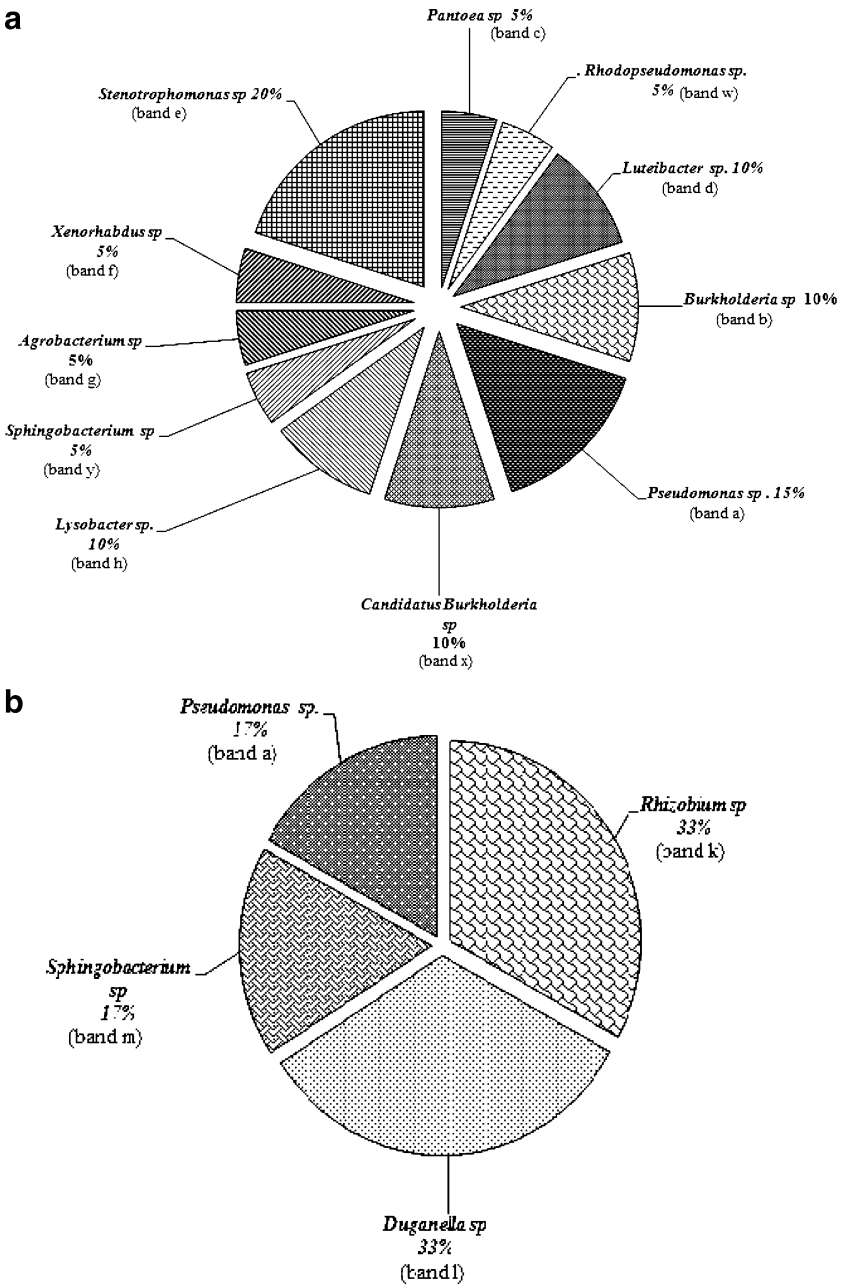

Figure 7 Taxonomic affiliations of metabolically active microbiota identified from the most preferred CLPP substrates after PCR-DGGE analyses. Shown are **a** un-mined site, **b** 1987 rehabilitated site, **c** 1997 rehabilitated site, **d** 2007 rehabilitated site

labile organic carbon pool in soils [9, 10, 27] and is considered a good indicator of soil recovery [1]. Several reports have previously indicated that reclaimed environments take several decades to reach levels of MBC found in undisturbed soils [2, 17, 27, 28]. Moreover, recovery of MBC is to some degree, site specific making it difficult to predict the rate of recovery for any given reclaimed area [1]. This is consistent with our observations of site CC24 rehabilitated in 1987, which was composed extensively of rocky terrain and sparse vegetation. It is likely that the excessive rocky soil structure combined with a semi-hilly terrain resulted in surface runoff of nutrients and loss in soil productivity of the 1987 rehabilitated site.

Moreover, soils are typically heterogeneous with irregular distribution and functions of microbial communities [8]. To gauge impacts of bauxite mining and restoration success, we then utilized CLPPs followed by PCR-DGGE identification of metabolically active microbiota from the most preferred substrates. Across all soils, carbohydrates were the most preferred substrates over amino acids, polymers, carboxylic acids and esters. Additionally, CLPP assays clearly discriminated between the un-mined and reclaimed soil microbial responses such that the un-mined site showed the highest AWCD and functional diversity based on the *H* and *E* indices, compared with the chronosequence soils from 2007 and 1987 (Table 2,

Figure 7 continued

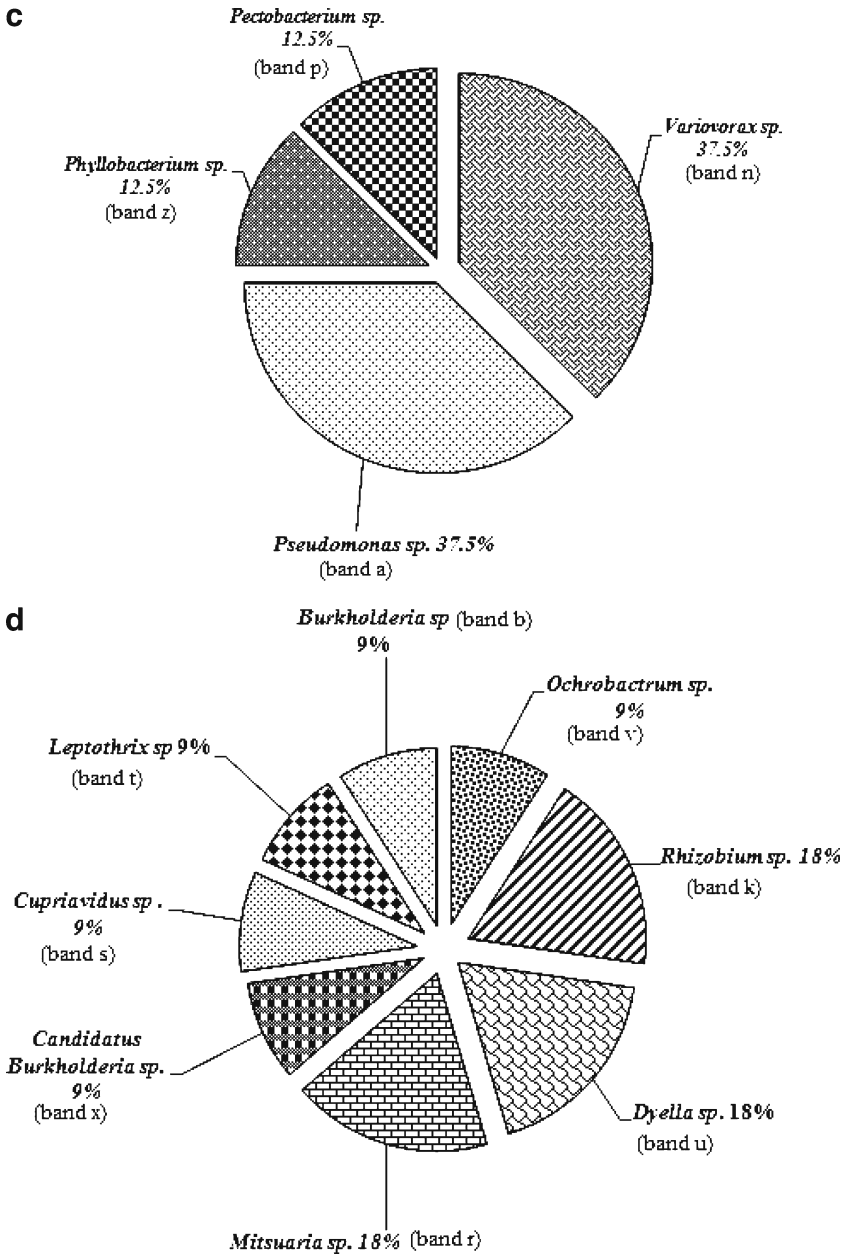

Fig. 2). Conversely, among the three rehabilitated soils, only those from the 1997 group (wet and dry seasons) resembled the physiological profiles of un-mined soils. This is in good agreement with the microbial biomass carbon values from this site, suggesting that this particular site may be further along on recovery than the other sites.

Overall, our results are in accordance with those from several other studies showing that environmental perturbation can result in severely altered ecological processes [15–17, 27, 28].

We recognize limitations associated with CLPPs such as not all soil microbes are readily cultivable and able to reduce the tetrazolium dye in the BIOLOG EcoPlates [38]. Nevertheless, our study shows that CLPP coupled with molecular analyses of the functionally active microbiota can be used as a sensitive indicator of soil functional activity [15].

Further, microbial diversity is a function of the environment and can provide for a sensitive assessment of rehabilitation processes [5, 6, 9]. DGGE analyses of the bacterial diversity from the most preferred CLPP substrates revealed further evidence of the differences within the bauxite soils. Specifically, the total numbers of DGGE bands from the un-mined sites were approximately 3-fold higher compared with that of the rehabilitated soils (data not shown). SSU rDNA analyses and sequencing of the predominant DGGE bands revealed that the metabolically active microbiota clustered under two different phyla-Proteobacteria and Bacteroidetes (Fig. 6; Supplementary

Table 1). Proteobacteria represents a metabolically and physiologically diverse group of bacteria and are of major significance to the cycling of nutrients [34]. Among the Proteobacteria, γ-proteobacteria was found to be the most abundant in our study. Gamma-proteobacterial lineages being opportunistic are known to have a propensity to respond rapidly to labile soil carbon [30, 32] and ephemeral nutrient pulses in aquatic systems [3]. It is very likely that low representation of γ-proteobacteria in the rehabilitated sites results in the low productivity of such perturbed soils.

This is one of a few multipronged studies to gauge the effects of mining perturbations on bacterial community structure and impending loss in soil productivity. Overall, the results presented here indicate that the rehabilitation of mined environments are complex and influenced by both, abiotic and biotic factors. However, the observation of distinct bacterial communties and activities in un-mined and mined soils suggest that the use of microbially mediated analyses can enhance assessment of rehabilitation processes. As pointed out in a recent article on restoration ecology, the critical question is whether soil microbiota serve as pioneer organisms and facilitate the recovery processes or merely colonize the niches during the slow reestablishment of higher life forms [19]. Our future studies investigating the restoration trajectory in Jamaica will likely provide an improved understanding of soil bacterial community succession and environmental productivity.

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