

Application of Emerging Tools and Techniques for Measuring Carbon and Microbial Communities in Reclaimed Mine Soils

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ABSTRACT / As part of a study of the potential for carbon sequestration in degraded mine lands, we examined the carbon content of reclaimed mine soils treated with soil amendments (e.g., fly ash and biosolids) using two emerging techniques; terminal restriction fragment-length polymorphism

(TRFLP) and laser-induced breakdown spectroscopy (LIBS). Both of these techniques have potential use for measuring aspects of carbon content and its role in the soil ecosystem. To better understand the relationship between the microbial community and the amount of carbon within mine soils, we examined the diversity among fungal communities in soils with different carbon content using TRFLP. TRFLP was run on 18S rDNA from polymerase chain reaction (PCR) amplification using primers specific for fungi. Results from the TRFLP were compared to sequencing of 18S clones. The diversity based on sequence analysis was much higher than that indicated by the TRFLP-based analysis. Rarefaction analysis of the data indicated that the total diversity was even higher than we were able to measure with both levels of effort; however, it was clear that we effectively sampled the dominant populations. The LIBS technique displayed a strong linear relationship when compared to conventional techniques (LECO and Walkley-Black) of measuring carbon in soils. In addition, discrepancies were noted between the two conventional techniques for soils with high carbon content.

Rising atmospheric CO₂ levels coupled with implications for global climate change have led to interest in the potential to store or sequester carbon in terrestrial environments (e.g., Lal and others 1999; Eve and others 2002; Bruce and others 1999). One approach to terrestrial carbon sequestration is to make use of currently underutilized and degraded lands. Nearly 2×10^9 ha of land are considered to be degraded worldwide (Lal and others 1998). In the United States alone, $\sim 4 \times 10^6$ ha consist of degraded lands previously used for mining operations (USDA 1979). Based on the 1.4×10^8 ha of poorly managed lands estimated for the United States (Oldeman and Vanengelen 1993) and estimates of carbon sequestration within degraded lands (i.e., 1.5 Mg C/year/ha) derived by Akala and Lal (2000), there is a potential for degraded lands in the United States to sequester ~ 11 pg of carbon over the next 50 years. However, the ability to obtain the maxi-

mum potential carbon sequestration on degraded lands may rely upon improvements in the soil through applications of soil amendments. Thus, investigations of amendments and their effect on soil carbon are needed.

Landowner decisions on the feasibility of a carbon sequestration project will depend on many factors (Batjes 1999). Two of these will be the cost of measuring the soil carbon and the decision on what amendments should be used. A critical challenge in the use of terrestrial carbon sequestration to meet international accords is the measurement of carbon in soil (Bowman and others 2002; Bruce and others 1999; Potter and others 1999; Ellert and others 2002). Accurate and efficient measurement of carbon in soil is critical to ensuring confidence in the process, but the cost must be moderate or the economic benefits of the carbon sequestration may be lost to the landowner. The decision on what amendments to use will be influenced by many factors, such as emissions of other greenhouse gases and the potential for leaching of pollutants from the amendments, in addition to the effect on soil carbon. Changes in the microbial community associated

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with amendments may give early indications of the eventual carbon sequestered in the soil, the potential for emission of other greenhouse gases (e.g., methane and nitrous oxide), and the potential toxicity of the amendment. Novel techniques for measuring both carbon content and microbial diversity have recently become viable options that can provide accurate measurements relating to a multitude of applications for determining the potential for carbon sequestration in soils.

Clearly, better techniques are needed for complete and efficient characterization of soil communities. Standard plating methods used in the past have proven to be relatively easy techniques for microbial analysis. However, there remains a large fraction of microorganisms that are not culturable, thus leaving an incomplete picture of community representation (e.g., Liu and others 1997). The use of advanced molecular techniques involving the extraction and sequencing of DNA has overcome many of these discrepancies, although they are rather time-consuming for community characterization. Research utilizing rDNA restriction analysis has provided an alternative and effective method for studying microbial communities (Smit and others 1999; Liu and others 1997; Borneman and Hartin 2000; Klamer and others 2002; Taylor and others 1999; Buchan and others 2002). We investigated the relationship between the microbial community and the carbon content of lands that had been mined by using terminal restriction fragment-length polymorphism (TRFLP) on 18S rDNA using primers specific for fungi. The results from the TRFLP analysis were then compared with those of cloning techniques for a measure of fungal biodiversity.

Conventional techniques of measuring carbon content in soil may not be cost-effective for sequestration projects (Lal 2002; Watson and others 2000). Techniques often require sample combustion and CO₂ gas analysis with lengthy preparation and analysis in a laboratory. Thus, the soil carbon analysis of the numerous samples that may be required to characterize changes in soil carbon for sequestration projects could be very expensive. Therefore, we examined the use of laser spectroscopic technique for carbon and nitrogen analysis because of its real-time monitoring capabilities, its high degree of analytical sensitivity and selectivity, and its potential use in a field setting (Martin and others 2003). Such qualities make it a good candidate for environmental analysis. To determine the applicability of such a novel technique, we compared the carbon and nitrogen content from mine soils determined from laser-induced breakdown spectroscopy (LIBS) with re-

sults from standard laboratory-based methods on the same mine soil material.

Materials and Methods

Experimental Design

To gain a better perspective of the enhancement of carbon content established in previously mined soils, we collected all soil samples for both microbial and carbon analysis from sites that had received different amounts of soil amendments. These amendments included varying combinations of sawdust, fly ash from fossil fuel combustion, and biosolids. Fly ash was chosen because of its potential to supply base cations and trace elements to mined lands, which generally have a low pH (Crews and Dick 1998). The benefits of fly ash incorporation include increased aggregation, porosity, water-holding capacity, pH, conductivity, and basic cations (Sale and others 1997; Matsi and Keramidas 1999). Sawdust and biosolid amendments were chosen because of their potential to increase organic matter within the soil, thereby creating a more reactive framework for water, air, and nutrients within the soil. In addition, biosolid amendments could also help stabilize toxic metals in the soil, reducing their uptake by plants and migration into groundwater.

Twenty-two soil samples were collected for geochemical and microbial analysis in June 2001 from four separate research plots established on reclaimed mine soils located in Wise County, Virginia, and Morgantown, West Virginia. Research plots varied in their establishment time and amendment applications. Table 1 provides a brief description of each plot according to amendment, land use, and sampling depths.

Study Areas

Controlled overburden placement experiment: Surface amendment. The Powell River Project CP research area, originally established in 1982 for research use by Virginia Tech, is located on the Powell River Project, in Wise County, Virginia. The research site exists in an area that includes reclaimed and abandoned mined lands and is also the site of an active mining operation (Haering and Daniels 2000). Four backhoe pits were dug in the surface amendment areas and four samples were collected for microbial analysis from the soil A horizon. Control plots consisted of 2:1 sandstone:siltstone, amended with 1120 kg/ha of 15-30-15 N-P-K fertilizer. Plots amended with sawdust consisted of 112 Mg/ha sawdust + 1120 kg/ha 15-30-15 N-P-K fertilizer + 336 kg/ha N fertilizer. Local municipal biosolid material was used to amend plots for organic biosolid application.

Table 1. Description of research plots

Site	Location of site	Pit ID	Amendment type	Amendment amount	Sample depth (cm)	Land use (forest, farm, etc.)
2	Wise Co., VA	PRS-D-Wet	Biosolid	368 Mg/ha	0–14	Pasture
3	Wise Co., VA	CP-48	Biosolid	224 Mg/ha	0–12	Pine forest
4	Wise Co., VA	PRS-PL-Control	Control	0	0–12	Pasture
7	Wise Co., VA	CP-43	Control	0	0–13	Pine forest
11	Morgantown, WV	Walls Farm 1	Fly ash	30.48 cm	8–22	Pasture
13	Morgantown, WV	Walls Farm 1	Fly ash	30.48 cm	0–8	Pasture
14	Morgantown, WV	Walls Farm 1	Fly ash	30.48 cm	60–80+	Pasture
15	Morgantown, WV	Walls Farm 2	Fly ash	30.48 cm	0–9	Pasture
16	Morgantown, WV	Walls Farm 2	Fly ash	30.48 cm	9–25	Pasture
20	Morgantown, WV	Jenkins Farm 1	Fly ash/sawdust	8–15 cm	0–7	Pasture
21	Morgantown, WV	Jenkins Farm 1	Fly ash/sawdust	8–15 cm	50–85	Pasture
23	Morgantown, WV	Jenkins Farm 2	Fly ash/sawdust	8–15 cm	0–9	Pasture
24	Morgantown, WV	Jenkins Farm 2	Fly ash/sawdust	8–15 cm	9–46	Pasture
25	Morgantown, WV	Jenkins Farm 2	Fly ash/sawdust	8–15 cm	46–80+	Pasture
26	Morgantown, WV	Jenkins Farm 3	Control	0	0–5	Pasture
27	Morgantown, WV	Jenkins Farm 3	Control	0	5–28	Pasture
30	Morgantown, WV	Jenkins Farm 4	Control	0	0–6	Pasture
31	Morgantown, WV	Jenkins Farm 4	Control	0	6–40	Pasture
32	Morgantown, WV	Jenkins Farm 4	Control	0	40–98+	Pasture

Powell River Biosolids (PRS) Project. The PRS project for biosolid demonstration was established in 1989 and was located on the Powell River Project in an area consisting of 70 ha of nontopsoiled mine soil amended with biosolids known as “Philadelphia mine mix.” Amendments were applied in a ratio of 1:1 biosolid: composted wood chips (Haering and Daniels 2000; Daniels and Haering 1994). Four backhoe pits were dug in the surface amendments for characterization of soil horizons and four samples were collected for microbial analysis from the soil A horizon.

Jenkins Farm and Walls Farm fly ash amended plots. Research plots from the Jenkins Farm area, established in 1989, are located in Preston County, West Virginia. Jenkins Farm sites were sampled on a 20% slope; this field site was originally designed for erosion studies. Fly ash from the nearby Albright Power Station was used for applications that consisted of a fly ash/sawdust (80:20 by volume) amendment. Four backhoe pits were dug and six samples were taken for microbial analysis for the top three soil horizons in the amended plots. In addition, five samples were taken from the top three soil horizons in control plots.

Walls Farm research plots, located near Morgantown, West Virginia, were established 30+ years ago. Five samples were collected for microbial analysis from the Walls Farm fly ash plots. These plots were amended with 30.48 cm of fly ash. Both A1 and A2 horizons were sampled from two separate research areas; in addition, the lower C2 horizon was sampled from site 1 of Walls

Farm. Samples were collected from a wall facing within an eroded gully.

Soil Collection

Samples for soil horizon characterizations were collected from backhoe pits, placed in gallon-size plastic bags, and transported on ice back to the laboratory. Approximately 2- to 3-g samples for microbial analysis were placed in separate small Whirlpak bags, wrapped in aluminum foil, and then placed into a liquid-nitrogen carrier for transportation back to the laboratory. Samples for soil horizon characterization were air-dried in the laboratory prior to analysis.

Molecular Methods

DNA extraction and quantification. Total nucleic acids were extracted from 1 g of soil recovered from the 22 sites for community DNA analysis (Hurt and others 2001). For DNA concentration, the samples were diluted with PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, Oregon) and were analyzed using Fluroreskan Ascent (ThermoLabSystems, Helsinki, Finland).

Primers, amplification, and TRLP analysis. Primers NL1 and 5' FAM NL4 (QIAGEN, Valencia, California) (Table 2), which amplify ~560 bp of the fungal V2/V3 (26S-635) region (White and others 1990), were used to duplicate 3'-end-labeled community fungal polymerase chain reaction (PCR) product libraries from 25-ng DNA samples recovered from 22 sites. The DNA ampli-

Table 2. Primers and sequences used for fungal community analysis

Primer	Sequence	Region	Ref.
NL1	5'-gca tat caa taa gcg gag gaa aag-3'	26S-635	White and others (1990)
NL4	5'-(6-FAM) ggt ccg tgt ttc aag acg g-3'	26S-635	White and others (1990)
FUN18S1	5'-cca tgc atg tct aag wta a-3'	18S-ITS	Reimers (2001)
FUN18S2	5'-(5-HEX) gct gga cca gac ttg ccc tcc-3'	18S-ITS	Reimers (2001)
EF4	5'-gga agg g[g/a]t gta ttt att ag-3'	18S-rDNA	Smit and others (1999)
EF3	5'-tcc tct aaa tga cca agt ttg-3'	18S-rDNA	Smit and others (1999)
T7	5'-aat acg act cac tat agg gc-3'	Bases 362–381	Invitrogen, Carlsbad, CA
M13 reverse	5'-cag gaa aca atg ac-3'	Bases 205–221	Invitrogen, Carlsbad, CA

fication was performed in 2.5 mM MgCl₂, 1X PCR buffer, 0.4 mM dNTPs, 1 mg/mL bovine serum albumin (BSA) (Promega, Madison, Wisconsin), and 2.5 units of *Taq* DNA polymerase. Thermal cycling was performed using a 2-min initial disassociation at 95°C followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min in a GeneAmp 9700 (Applied Biosystems, Foster City, California). PCR products were pooled, ethanol-precipitated, and dissolved in 25 µL H₂O. Aliquots (6 µL) of the labeled DNA were used for digestion containing 1 unit each of restriction endonuclease *Msp*I and *Hha*I, respectively, at 37°C for 4 h (Invitrogen, Carlsbad, California). The digested fragments were diluted 1:6 in double-deionized water (dd H₂O) and then further diluted 1:10 in Hi-Di formamide (Applied Biosystems, Foster City, California) that included GeneScan 400HD [ROX] Size Standard (Applied Biosystems). The fragments were then electrophoresed on an ABI Prism 3700 DNA analyzer (Applied Biosystems). The peaks were analyzed using Genotyping software (Applied Biosystems).

Primers FUN18S1 and 5' HEX-labeled FUN18S2 (Reimers 2001) were used for analysis of the divergent region of the fungal 18S ribosomal sequence (Table 2). PCR conditions were as described earlier.

Cloning and sequencing analysis of 18S ribosomal genes. Seven sites were chosen for preparation of 18S ribosomal gene libraries constructed using primers EF4 and EF3 (Smit et al 1999). The 1.4-kb 18S PCR products were gel-purified using a Wizard PCR Purification System (Promega, Madison, Wisconsin) and were ligated into pCR 2.1 vector (Invitrogen, Carlsbad, California). The plasmids containing 18S ribosomal DNA were purified using Montage Plasmid Miniprep96 kit (Millipore, Bedford, Massachusetts) and were sequenced using Big Dye terminator Ready Reaction mix (PE Applied Biosystems, Foster City, California). The reactions were prepared using 2 µL Big Dye, 2 µL of 5X sequencing buffer (400 mM Tris-HCl pH 9.0, 10 mM MgCl₂), 4 µL plasmid DNA, 10 pmol, primer T7, M13 reverse primer, or EF4 (Table 2), in a 10-µL reaction

volume. Cycle sequencing on the Gene Amp 9700 included 35 cycles of 95°C for 15 s, 50°C for 5 s, and 60°C for 4 min. Extension products were ethanol-precipitated by adding 8 µL water and 32 µL of 95% ethanol. The samples were run on an ABI Prism 3700 DNA Analyzer (Applied Biosystems) and were analyzed using the Sequencer sequence analysis software (Gene Codes, Ann Arbor, Michigan). The sequences were subjected to National Center for Biotechnology Information (NCBI) blast search to identify closest relatives.

The clone data were used to cluster sites based on the similarity of clones (McCallum and others 2001) present using PC-ORD (MJM Software Design, Gleneden Beach, Oregon). We used the Bray–Curtis method with Euclidean distance measures; however, the clusters were most robust when other approaches were used (PC-01).

Chemical Analysis

Carbon–nitrogen analysis. In the combustion method, 0.5–0.6 g of soil were weighed in a ceramic sample boat. The sample was inserted into the combustion chamber in a LECO® CN-2000 elemental analyzer (LECO Corporation, St. Joseph, Michigan) which heats the sample to a temperature of 1350°C in the presence of oxygen. The combustion gases were analyzed for CO₂ (infrared spectroscopy) and N₂ (thermal conductivity detector). The elemental analyzer was calibrated with standards traceable to the National Institute of Standards and Technology (Gaithersburg, Maryland).

An additional conventional method, a modified Walkley–Black method with colorimetric analysis (Donahue and Heckendorn 1996; Nelson and Sommers 1996), was also used to determine organic matter from the soils.

Acid washing to remove inorganic soil carbon. Prior to analysis on the LECO and LIBS, subsamples of soil were taken from collection bags, milled, and put through a #30, 5-mm sieve. Inorganic carbon was removed from the sample through a series of hydrochloric acid washes so that soil organic carbon could be determined.

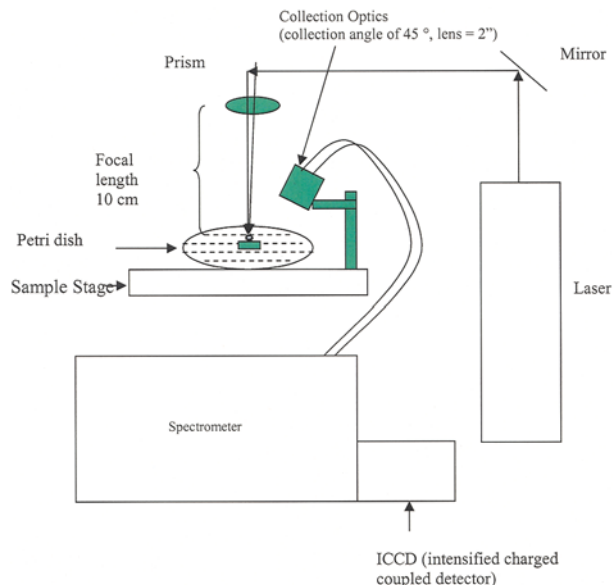


Figure 1. LIBS experimental setup for carbon and nitrogen detection.

Sample pellets used in the LIBS technique were formed from homogeneous soil samples using the following technique. The homogenized soils were weighed and placed in disposable glass culture tubes. Ultrapure deionized water (0.5 mL) was mixed with the 0.5-g soil sample tubes, and the mixture was heated at 60°C for 24 h or until dry. The tubes were then turned upside down and gently tapped to release the pellets. The pellets were stored in airtight vials to prevent absorption of moisture from the atmosphere (Martin and others 2003).

LIBS technique. The LIBS technique requires a pulsed laser as the excitation source. A plasma can be formed when a laser beam of sufficient energy is focused onto a small area, creating a power density inside the sampling volume ($\sim 14 \times 10^{-9} \text{ cm}^3$) exceeding tens of gigawatts per square centimeter. This heating vaporizes material in the focal volume into its elemental constituents. This plasma appears as a spark accompanied by an audible crack as the plasma expands outward at supersonic speed in all directions, with an initial rate of expansion on the order of 10^5 m/s . The experimental setup used to determine the concentration of carbon and nitrogen in soils is shown in Figure 1.1 In the experimental configuration, we use a Spectra Physics model Indi-HG laser that is a Q-switched Nd:YAG laser with output wavelengths of 1064, 532, and 266 nm. The laser was used at 266 nm with typically a 23-mJ/pulse. The light emitted by the plasma at the focal volume was collected by a set of collection optics and was focused into a low O-H silica fiber bundle

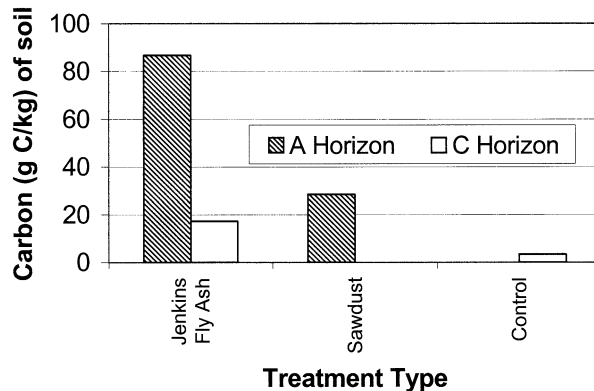


Figure 2. Carbon concentration (g C/kg soil) amounts determined by amendment treatment type for controlled placement experimental plots.

consisting of 19 fibers. This fiber bundle (numerical aperture [NA] = 0.22, diameter = 4.66 mm) delivered the light to a 0.5-m Acton Research model SpectraPro-500 spectrometer (spectral bandwidth = 40 nm for 1200-g/mm grating and slit width of spectrometer = 25 μm), which was then detected by an intensified charge-coupled detector (ICCD) (Andor Technology, South Windsor, Connecticut). The advantage of using an ICCD is that the detection can be delayed with respect to plasma formation. In addition, the detector can be gated to prevent high-intensity background light from the plasma in its early stages of formation from entering the detector. Thus, gating and delay of the gate serves to optimize the signal-to-noise ratio (S/N) of the acquired spectrum. The smallest gate width that the ICCD detector can achieve is 2.2 ns. Thus, time-resolved laser-induced breakdown spectroscopy was used (Martin and others 2003).

Results and Discussion

Comparison of Carbon Measurement Techniques

Apparently due to the variations in treatments and the depths of the soil sampled (Figure 2), we generated a fairly large range in soil carbon (less than 2 to $\sim 80 \text{ g C/kg}$ soil in individual replicates) over which to generate and compare the LIBS technique to the combustion methods (Walkley-Black and LECO). Soil carbon measured by the LIBS technique exhibited a strong linear relationship for the previously mined soils when compared with the content measured by the LECO gas combustion technique (Figure 3, inset) and the Walkley-Black method over the range of carbon up to $\sim 60 \text{ g C/kg}$ soil. However, a sample at higher carbon (82.5 g C/kg soil) did not fit the regression (Figure 3, inset).

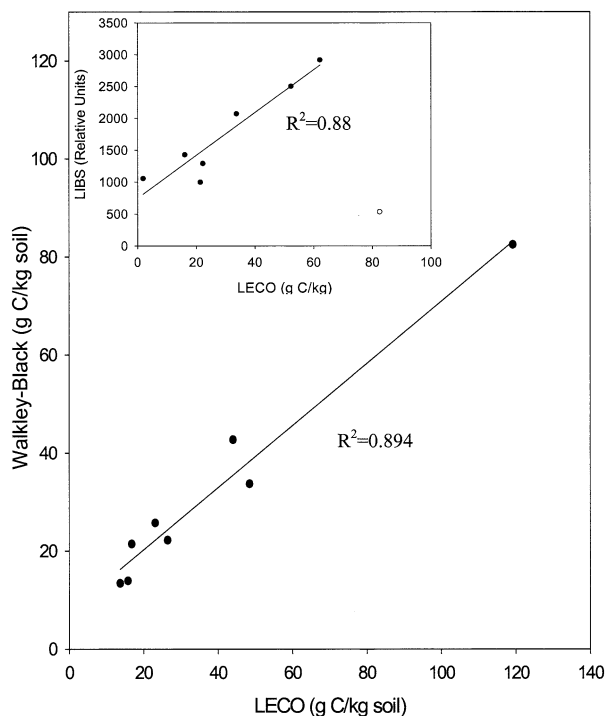


Figure 3. Walkley–Black method compared to LECO gas combustion method for previously mined soils and LECO carbon calibration of LIBS data (inset). Discrepancies appear among the methods in these soils above 60 g C/kg soil (as measured by LECO) where the LECO and Walkley–Black fit becomes nonlinear (e.g., poor fit of regression at high carbon) and the LECO calibration fails (open circle in inset).

Comparisons of the LECO technique and the Walkley–Black method appeared to have a nonlinear relationship at high amounts of carbon (Figure 3). Apparently, there are discrepancies among the methods at high levels of carbon in soils. Of the original 22 sites collected, data from several sites was removed because we could not achieve good data extraction.

To achieve these results with the LIBS technique, it is advisable to make a large number of measurements. For example, although the range can be substantial, approaching 50% of the mean for some samples (e.g., Figure 4), standard error of the measurement is quite small ($\sim 3\%$) when 20 measurements are made on a soil sample (Figure 4). Because it is quite easy to make this number of measurements in a small period of time, small measurement errors are easy to achieve. Thus, the major source of error is likely to be sampling error, which must be addressed by taking multiple samples within a plot.

The best strategy for applying LIBS to measurements intended to support claims of carbon sequestration will likely be different from a strategy that would best mea-

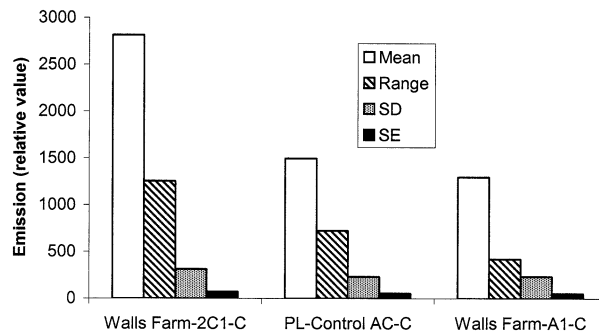


Figure 4. LIBS measurements on 20 soil samples from previously mined soil.

sure the carbon content in a single soil sample. A carbon assessment strategy would likely be based on application of the technique to a relatively large number of samples from a plot with a small number of subsamples (e.g., pellets).

Microbial Analysis

TRLFP results. A number of parameters in the application of TRFLP need to be set to run the analysis; the first stage of this work involved selection of a method and development of parameters that would allow relatively easy and automated treatment of the data. Thus, we chose to use presence and absence of a fragment rather than attempting to quantify each fragment (Horton 2002; Timonen and others 1997). We also chose to establish a peak-height criterion for the selection of fragments to include in the analysis. We analyzed peaks with peak-height criteria of 70, 250, and 500 and found strong linear relationship (significance $> 95\%$) among these (for setting 70 versus 250, $R^2 = 0.79$; for setting 70 versus 500, $R^2 = 0.58$; for setting 250 versus 500, $R^2 = 0.82$). The lowest ratio of mean number of fragments to the standard deviation of the number of fragments was found with the most sensitive setting of 70. This still gave us a reasonable number of peaks to use in the analysis.

The number of fragments generated using the technique and potentially the patterns generated are also going to depend on the regions amplified and the restriction enzymes used. Results using primers set for the 18S ITS region and the 26S-635 V2/V3 regions were compared, and, as expected, the ITS primer yielded greater variability and a greater number of fragments as well as a wider range of fragments among sites (Table 3). Thus, for subsequent analysis, we used those primers. Examination of plots of the number of fragments generated with the ITS region using either *MspI* and *HhaI* did not indicate a simple relationship, as there was a low correspondence between them at given sites.

Table 3. Comparison of TRFLP primer sets for 18S ITS region and 26S-635 V2/V3 region fragments

Primer set	Mean No. of fragments	Standard deviation of No. of fragments	Maximum No. of fragments	Minimum No. of fragments
18S ITS	25.7	14.7	65.0	1.8
26S-635 V2/V3	17.9	9.1	50.0	2.0

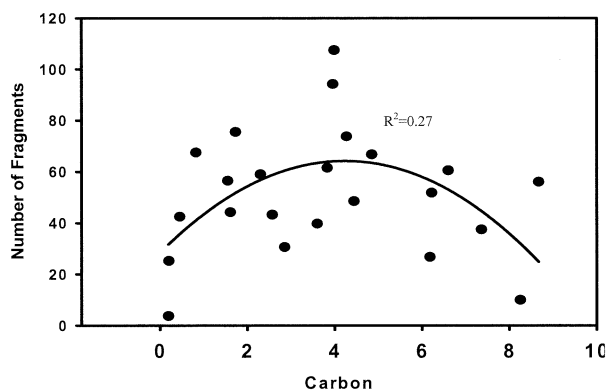


Figure 5. Number of fragments (ITS region) determined by carbon content in soils. Nonlinear regression was significant at the 95% level ($R^2 = 0.27$).

It appeared that the utilization of the different enzymes might have generated information on different portions of the microbial population. Consequently, we used fragments generated with both sets of restriction enzymes in the analysis.

Using a peak-height criterion of 70 and primers for the ITS region and summing the fragments from both sets of enzymes, we observed the greatest amount of molecular diversity (number of fragments) at sites containing intermediate carbon levels (Figure 5) and intermediate nitrogen levels (not shown). We observed a very similar relationship to nitrogen ($R^2 = 0.28$ at 95% confidence level). The low R^2 derived from a second-order (nonlinear) fit of soil carbon content and nitrogen content to the number of fragments present likely indicates the presence of additional factors that could be considered important in fungal diversity.

Thus, it appears ITS-TRFLP can be a useful method as a measure of diversity, but it does not provide adequate taxonomic information. Our results are comparable to Horton (2002) insofar that ITS-TRFLP data provided limited taxonomic information and that direct sequencing or phylogenetic analysis should be conducted in order to increase the information gleaned from taxonomic levels. Further complications involving the use of TRFLP include the possible coexistence of multiple ITS regions within single species (Sanders and others 1995, 1996).

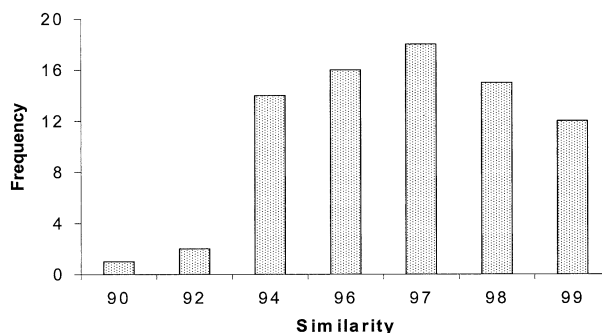


Figure 6. Similarity of cloned 18S sequences to sequences found in the database.

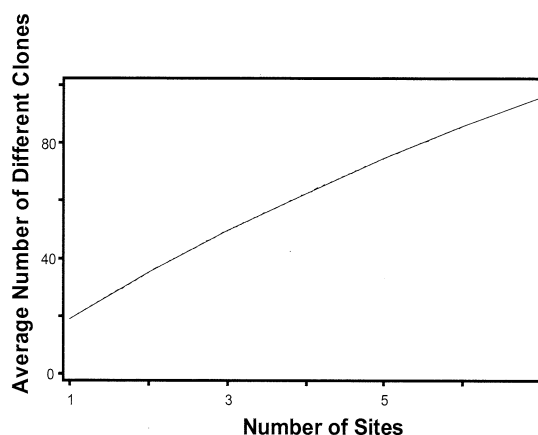


Figure 7. Rarefaction analysis of species area curve (including all sites).

Cloning results. Information gathered from cloning was used in a phylogenetic approach to match databases for measures of diversity and to explore the relationships to the amendment treatments to carbon. Results of the cloned 18S sequences indicated that very few clones were highly divergent from the database search (Figure 6). Many clones were greater than 97% similar to anything in the database. Smit and others (1999) also observed many 18S rDNA fungi clone sequences that were similar to those in the RDP database, but they also observed a much higher proportion with similarities of less than 90%. Cloning and sequencing indicated that, along with the few dominant populations,

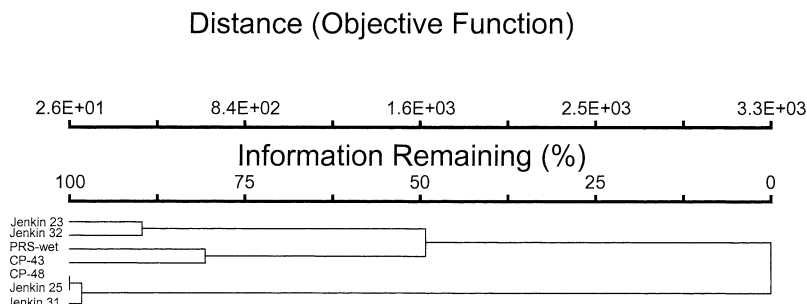


Figure 8. Cluster analysis of species data.

there was a surprising diversity of fungal clones. This measured diversity was higher than that indicated by the TRFLP analysis. Rarefaction analysis of the data indicated that the total diversity of fungal clones was even greater than we were able to measure with this level of effort (Figure 7); however, it appeared that we were able to effectively sample the dominant populations.

The five most dominant populations (> 50%) included the following: *Mortierella verticillata*, a common acidophilous fungus in forest soils of temperate zones, the litter of pine and spruce trees, peat bogs, and sand dunes; *Sordaria fimicola*, a common coprophilous pyrromycete found on fresh droppings of many herbivorous animals in forest soils and grasslands; *Mortierella chlamydospora*, generally found in agricultural fields and capable of decomposing chitin (Domsch and others 1980); *Cryptococcus humicolus*, which has been isolated from acidic sandy soil supplied with benzene compounds and coke-plant wastewater (Middelhoven and others 1992; Kwon and others 2002); and *Cercophora septentrionalis*, which is often found on submerged wood (Domsch and others 1980). Sequences for *Sordaria fimicola* were found by Smit and others (1999) when cloning and sequencing from a wheat rhizosphere, as were species of *Mortierella* and *Cryptococcus*. The > 50% pattern of sequences coming from only five clones is quite different from that observed in surface soils for bacteria where the most dominate populations account for just a few percent of the total (e.g., Zhou and others 2002). However, Buchan and others (2002) observed this dominance by a few cloned types for ascomycete fungi on *Spartina alterniflora* blades in a salt marsh.

Ordination techniques used to assess relationships among the sites in terms of fungal community structure revealed little consistency among samples from the same treatment (Figure 8). One possible explanation is that because cloning and sequencing techniques are, of necessity, done on only a small portion of the genetic material in the samples, the samples do not represent the community well. A potential approach to obtain

more detailed information on diversity and the functional capabilities over a wide range of sites is the utilization of microarrays (e.g., Wu and others 2001) that would assess 10^6 – 10^8 more genomes in each measurement than is feasible with cloning and sequencing. Microarrays would sample a much larger portion of the population and could be applied to more sites.

We will be using the clone data to design an array to be utilized on these sites. The adequacy of the array will depend on our inclusion of enough relevant species information from the cloning. The top 14 clones observed at the 7 sites represented 69% out of the total 405 sequenced. We should be able to utilize this information to design a small array. Ten of these 14 appear at more than 1 site. The next nine most numerous clones represented 6.7% of the total clones. An array using many of the common species found in the clone libraries and a selection of relatively uncommon species is being tested and compared with the TRFLP and cloning approaches. It also includes hundreds of genes for bacterial functional genes. Thus, we hope to determine whether the differences in carbon content related to the original treatments (e.g., fly ash and biosolids) are related to differences in the microbial community that can be assayed with this combined functional and phylogenetic array.

Conclusions

The LIBS technique, used on soils before and after acid-washing, appears to be useful for the determination of both organic and inorganic soil carbon. Strong linear correlations were obtained between standard laboratory-based techniques and LIBS; thus, the LIBS technique has the potential to be packaged into a field-deployable instrument for in situ, real-time analysis of carbon.

Although relationships to important parameters were established with the TRFLP data, cloning and sequencing of 18S give more detailed data. However, the data are still limited to phylogenetic information and

small numbers of samples. The use of microarrays may yield both phylogenetic and functional information that will help us better establish the relationship between the microbial community and carbon sequestration.

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