Soil Biology & Biochemistry 46 (2012) 63–72

Soil Biology & Biochemistry

Pyrosequencing and mid-infrared spectroscopy reveal distinct aggregate stratification of soil bacterial communities and organic matter composition

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ARTICLE INFO

Article history:
Received 23 August 2011
Received in revised form
16 November 2011
Accepted 17 November 2011
Available online 2 December 2011

Keywords:
Pyrosequencing
Soil bacterial communities
Aggregate-size fractions
Mid-IR spectroscopy
UniFrac

ABSTRACT

This study integrated physical, chemical, and molecular techniques to assess relationships between soil bacterial community structures and the quantity and quality of soil organic carbon (SOC) at the soil microenvironment scale (e.g., within different aggregate size-fractions). To accomplish this goal, soil samples (0–5 cm) were collected from the Texas High Plains region under a variety of dryland and irrigated cropping systems. The soil was separated into macroaggregates, microaggregates, and silt + clay fractions that were analyzed for (1) bacterial diversity via pyrosequencing of the 16s rRNA gene and (2) SOC quantity and quality using a combustion method and mid-infrared diffuse reflectance spectroscopy (mid-IR), respectively. Results from pyrosequencing showed that each soil microenvironment supported a distinct bacterial community. Similarly, mid-IR data revealed distinct spectral features indicating that these fractions were also distinguished by organic and mineral composition. Macroaggregates showed relatively high abundance of *Actinobacteria* (excluding order *Rubrobacteriales*) and *α-Proteobacteria* and contained the most SOC. Microaggregates showed high relative abundance of *Rubrobacteriales* and the least amount of SOC. Predominance within the soil microenvironment and correlations along the mid-IR spectra were different between members of the order *Rubrobacteriales* compared with all other members of the *Actinobacteria* phyla, suggesting they have different ecological niches. Mid-IR results revealed microaggregates had greater absorbance in the 1370–1450 cm^{-1} region for phenolic and alkyl groups (possibly recalcitrant C). Silt + clay fractions were distinguished by *Gemmatimonadetes* and OP10 phyla, which positively correlated with spectral absorption in the1250–1150 cm^{-1} range (indicating both degradable and recalcitrant C forms). In contrast to general diversity index measurements, distributions of the more rare bacterial phyla (phyla representing <6% of the identified population) were more important for differentiating between communities in soil microenvironments. To our knowledge, this is the first study to investigate soil bacterial communities among soil aggregates using pyrosequenging and to associate these communities to specific soil C chemistries as indicated by mid-IR absorbance.

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The majority of soil microbial analyses are performed on bulk soil samples via methods that tend to standardize variability and result in a loss of information regarding the spatial [illegible] microorganisms and soil organic matter (SOM) (Mummey et al., 2006; O Donnell et al., 1995). Physical separation of the soil into definable aggregate fractions, such as initially proposed by Elliott (1986), and subsequent molecular analysis, allows one to investigate, at appropriate scales, the relationships between microbial communities and soil microhabitats. These approaches will enable investigators to link biotic dynamics with physicochemical information to provide a better understanding of soil functioning (Mummey et al., 2006).

An aggregate stratification strategy separates soil into at least three distinct microenvironments: macroaggregates (250 μm),

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doi:10.1016/j.soilbio.2011.11.012

microaggregates (53–250 μm), and silt + clay (<53 μm), which are differentiated by unique physical, chemical (organic matter and clay content) and structural (stability and size) characteristics (Ranjard et al., 2000). Macroaggregates protect plant-derived and microbial-derived SOM, contain high amounts of fungal biomass and are enriched with labile C and N originating predominately from plant residues. Microaggregates are formed by microbial induced bonding of clay particles, polyvalent metals and organo-metal complexes. They are characterized by lower concentrations of new and labile carbon (lower C:N ratio) and increased amounts of physically protected and biochemically more recalcitrant C compared to macroaggregates. The silt plus clay fractions are characterized by the highest concentration of relatively stable organic C and N, and they provide a protective environment for microbial biomass and microbial-derived SOM (Elliott, 1986). Despite the essential role of soil microorganisms in the formation and stabilization of soil aggregates, microbial community structure and composition within and among various soil aggregates is not well characterized (Mummey et al., 2006).

Once soil aggregates are physically stratified or fractionated, the challenge is adequately assessing soil biological and chemical components. Researchers have investigated soil microbial communities and activities within aggregates using methods such as phospholipid fatty acid (PLFA) profiles (Kong et al., 2011), fatty acid methyl ester (FAME) profiles (Schutter and Dick, 2002) and enzymatic assays (Allison and Jastrow, 2006). Differences in the distribution of bacterial species in different aggregate-size classes have also been revealed using molecular methods (Blackwood et al., 2006; Kabir et al., 1994; Ranjard et al., 2000). More recent work has used cloning and sequence analysis of 16S rRNA genes to elucidate the relationship between inner- and outer-aggregate fractions and microbial community structure (Mummey et al., 2006). Moreover, massive parallel sequencing, such as pyrosequencing now allows for the investigation of microbial diversity at levels previously not feasible (Roesch et al., 2007; Acosta-Martinez et al., 2008, 2010).

Quantification of soil organic C (SOC) is a relatively simple process, typically determined by combustion methodologies. However, characterization of the quality or chemical composition of this material is less straight-forward. A relatively novel technique employs mid-infrared (mid-IR) diffuse reflectance spectroscopy (DRIFTS) to acquire detailed information about the chemical composition of a soil. Calderón et al. (2011b) used such an approach on fractionated fresh and incubated agricultural soils to determine changes in SOM chemistry. Their results substantiated the importance of chemical changes in soil C and the use of this innovative tool for investigating different C cycling processes.

Soil microenvironments provide unique habitats for distinct microbial communities (Kong et al., 2011). Understanding the mechanisms that shape and influence soil microbial community structure, such as spatial segregation within aggregates, has important implications on the preservation of biodiversity and management of microbial communities for bioremediation, biocontrol of diseases and enhanced soil fertility (Grundmann, 2004). The objective of our study was to integrate physical, chemical, and molecular techniques to assess relationships between soil bacterial community structure and the quantity and quality of SOC at the soil microenvironment scale (e.g., within different aggregate-size fractions). To accomplish this goal, we fractionated soil samples into three aggregate-size fractions macroaggregates (>250 μm), microaggregates (53–250 μm), and silt + clay (<53 μm) and evaluated bacterial diversity via pyrosequencing. We also assessed SOC quantity using a combustion method and SOC quality using a mid-IR DRIFTS technique. We hypothesized that each aggregate-size fraction would contain a distinct bacterial assemblage and that these individual assemblages would be different in structure and composition from the bacterial community determined on whole soil samples. We further postulated that community composition within aggregates could be explained in part by soil chemical characterization unique to each aggregate-size fraction. We acknowledge that agricultural management practices are important drivers for soil microbial community dynamics and soil chemical composition, however, for the purpose of this study, we chose to focus only on the effect of aggregate-size.

2. Materials and methods

2.1. Agroecosystems description

Soil samples were collected from five agroecosystems representing long-term research and producer operations (>5 years at time of sampling). They included three integrated crop and livestock (ICL) systems and two cotton-cropping (CTN) systems located in the Texas High Plains region (THP). These agroecosystems (i.e., sites) are components of a larger study investigating the impacts of agricultural management on soil quality and economics (USDA-SSARE project numbers L597-82 and LS10-229). The specific management details for the five systems are presented in Table 1. Two of the ICL systems (#1 and #2) and one of the cotton systems (#3) are operated at the Texas Tech University Experimental Farm (TTEF) in New Deal, TX. Each system is replicated three times in a randomized complete block design. The other two agroecosystems, #4 and #5, are producer operated and are two of 27 fields, which are part of a long-term monitoring project named the Texas Alliance for Water Conservation (TAWC) program (TAWC, 2010).

The THP is characterized by a semi-arid climate, where average daily temperatures range from 7.9 to 22.9 °C (coolest in January with average − 4.4 °C, and warmest in July with 33.3 °C), with mild winters and a mean annual precipitation of 465 mm occurring mostly from April through October. All farming systems were established on Pullman clay loam soils (fine, mixed, superactive, thermic Torrertic Paleustolls) with an average of 38% clay, 28% silt, and 34% sand and a pH of 7.7 in the top 20 cm. Pullman soils, found on 1.5 million hectares of the Southern Great Plains, are of great agricultural importance and, thus, are considered a benchmark soil for this area (Unger and Pringle, 1981).

2.2. Soil sampling

Soil surface samples were collected in summer (July–August) 2010 from the five agroecosystems described in Table 1. From the replicated study at TTEF, one composite sample was taken from each of three field replicates of each agroecosystem evaluated. For the TAWC systems # 4 and #5, where traditional field replicates do not exist, three transects were established prior to sampling, marked by GPS, and five soil samples were collected along each transect and combined to create three spatially distinct field samples. The samples were sub-divided into 0–5 and 5–20 cm depths, but the 0–5 cm depth was used in this study. Within 48 h of sampling, field-moist samples were gently and thoroughly mixed and passed through an 8-mm sieve, with large aggregates being broken manually along planes of weaknesses (Six et al., 1998). Sieved samples were stored up to one week at 4 °C prior to fractionation and subsequent DNA extraction for pyrosequencing analysis.

2.3. Soil fractionation

Soil was fractionated using a wet-sieving technique based on that described by Elliott (1986) and Cambardella and Elliott (1993)

Table 1
Site descriptions of five agroecosystems in the THP. Values for soil pH and soil organic matter are reported as means for 0–5 cm with standard error in parentheses.

Agroeco-system[a]	Location	GPS coordinates	Crops/forages	Total area (ha)	Age (yrs)[b]	Cattle grazing	Irrigation type[c]	2010 Annual rainfall (mm)	Soil pH_{H_2O}	Soil organic matter (%)
1 (ICL)	New Deal, TX	33.733579°N, −101.738942°W	Native perennial forage[d]	13.5	6	Yes	None	679	7.2 (0.12)	2.4 (0.49)
1 (ICL)			Cotton (*Gosspium hirsutum* L.) (2010)/Millet (*Seteria italic*) (2009)	5.1	6	No	None		7.3 (0.09)	1.6 (0.09)
1 (ICL)			Millet (2010)/Cotton (2009)	5.1	6	Yes	None		7.0 (0.09)	1.7 (0.06)
2 (ICL)	New Deal, TX	33.733579°N, −101.738942°W	Bermudagrass (*Cynodon dactylon*)	2.7	6	Yes	SDI	679	7.6 (0.20)	2.7 (0.31)
2 (ICL)			OWB-B.Dahl (*Bothriochloa bladhii*)	6.3	6	Yes	SDI		7.6 (0.10)	2.2 (0.12)
3 (CTN)	New Deal, TX	33.732275°N, −101.736324°W	Cotton	0.25	5	No	SDI	679	8.0 (0.15)	1.9 (0.09)
4 (CTN)	Lockney, TX	34.104561°N, −101.447446°W	Cotton	41.6	5	No	SDI	722	7.5 (0.09)	1.5 (0.12)
5 (ICL)	Lockney, TX	34.133956°N, −101.480329°W	OWB-B.Dahl	11.2	5	Yes	CP-MESA	777	7.6 (0.07)	3.2 (0.27)
5 (ICL)			Corn (*Zea mays* L.) (2010)/Sunflower (*Helianthus annuus*) (2009)/Corn (2008)	44.7	5	No	CP-MESA		7.7 (0.03)	2.3 (0.06)

[a] ICL = integrated crop and livestock system; CTN = cotton-cropping system.
[b] Minimum age of site with detailed records.
[c] SDI = Subsurface Drip Irrigated; CP = Center-Pivot; MESA = Mid-Elevation Spray Application.
[d] Species include: blue grama (*Bouteloua gracilis*), sideoats grama (*Bouteloua curtipendula*), buffalograss (*Buchloe dactyloides*), and green sprangletop (*Leptochloa dubia*).

with the following modifications: soil was not air-dried before sieving; resulting fractions were not dried prior to DNA extraction; and the silt plus clay (SI + CL) fraction (<53 μm in size) was isolated via centrifuging (5000 rpm for 10 min) after addition of a weak $CaCl_2$ solution (0.01 ppm). In brief, 80 g of field-moist soil (<8 mm) was placed onto a pre-weighed 250-μm sieve and gently lowered into a basin filled with ultrapure water fitted with a DNAse/RNAse-free filter (Synergy® UV, Millipore, MA, USA) to allow the aggregates to become saturated. The basin was filled so that the water level would reach approximately 1 cm above the 250-μm sieve. After 5 min of soaking, the soil was sieved by moving the sieve up and down 50 times over 2 min (approximately 3 cm amplitude) under a slight angle to ensure that the water and small particles were able to pass through the sieve. After this 2 min sieving, the outside of the sieve was rinsed to ensure that no soil remained on the sides and weight of the wet sieve and soil was recorded. Soil remaining on the 250-μm sieve comprised the macroaggregate fraction (MACRO). This material was transferred to a sterile 50-ml centrifuge tube. Water and soil that passed through the 250 μm sieve were poured through a pre-weighed 53-μm sieve in a second basin, and the sieving procedure was repeated. Soil which remained on the 53-μm sieve comprised the microaggregate fraction (MICRO; 53–250 μm) and was transferred to another sterile 50-mL centrifuge tube. Sample remaining in the basin, which represented the SI + CL fraction (<53 μm), was treated with $CaCl_2$ to achieve a 0.01*M* concentration and transferred into 250-ml centrifuge bottles, centrifuged at 4 °C and 5000 rpm for 10 min. Free liquid was aspirated and the soil slurry was transferred into a sterile 50-mL centrifuge tube and stored at 4 °C until DNA extraction.

2.4. Bacterial community assessment: DNA extraction and pyrosequencing

The DNA was extracted from approximately 0.7 g of moist soil using the Fast DNA Spin Kit for soil (MP Biomedicals, OH, USA) according to manufacturer's instructions. The extracted DNA (1 μL) was quantified using an Epoch plate spectrophotometer (Biotech Instruments, VT, USA). The ratio of absorbance at 260 and 280 nm ($A_{260/280}$) was used to assess purity of nucleic acids and pure DNA $A_{260/280}$ was considered at or above ~1.8. All DNA samples were diluted to equivalent 30 ng/μL concentration for a 50-μl PCR reaction.

The 16S universal Eubacterial primers 530F (5′- GTG CCA GCM GCN GCG G) and 1100R (5′- GGG TTN CGNTCG TTG) were used for amplifying the ~600 bp region of the 16S rRNA genes. Sample DNA was amplified using a single step reaction (35 cycles) and 1U of HotStart HiFidelity Polymerase was introduced to each reaction (Qiagen). A two-region 454 sequencing run was performed on a 70 × 75 GS PicoTiterPlate (PTP) via Titanium sequencing platform (Roche, Nutley, New Jersey; Research and Testing Laboratory, Lubbock, TX). Sixty-four processed sequences acquired from the FLX sequencing run were further trimmed via a custom scripted bioinformatics pipeline (Handl et al., 2011; Ishak et al., 2011). In summary, each individual sequence was trimmed to a Q25 average, short reads <300 bp depleted, sequences with ambiguous base calls depleted, sequences with homopolymers exceeding 6 bp depleted, until only quality sequence information remained; tags were then extracted from the FLX-created multi-FASTA file while being parsed into individual sample specific files based on the tag sequence. Tags with less than 100% homology, compared to the original sample tag designation, were considered questionable in quality and were not considered. Based upon sequence identity, each bacterium was identified to its closest relative and taxonomic level. Sequence collections were depleted of chimeras using B2C2 software for batch depletion of chimeras from bacterial 16S datasets. Bacterial taxa were identified using Krakenblast (www.krakenblast.com) and compared to a custom highly curated database (Research and Testing Laboratory, Lubbock, TX). After the best hit processing, genus and higher level taxonomic designations were compiled using a secondary post-processing algorithm and relative percentages of bacterial taxa were determined for each individual sample.

2.5. Soil chemical composition as determined by mid-IR DRIFTS

Whole soil and soil aggregate fractions were scanned undiluted in mid-IR via Digilab FTS 7000 Fourier transform spectrometer (Varian, Inc., Palo Alto, CA) with a deuterated, Peltier-cooled, triglycine sulfate detector and potassium bromide beam splitter. The spectrometer was fitted with a Pike AutoDIFF diffuse reflectance accessory (Pike Technologies, Madison, WI) and potassium bromide was used as background. Data were obtained as pseudo-absorbance (log[1/Reflectance]). Spectra were collected at 4-cm^{-1} resolution with 64 co-added scans per spectrum from 4000 to 400 cm^{-1}.

2.6. Data analyses

Despite efforts to ensure high quality DNA for subsequent pyrosequencing analysis (e.g., quantification of DNA using

Nanodrop and 260/280 ratio analysis), only 84 of the 96 submitted samples were successfully amplified. Nevertheless, whole soil or aggregate fractions were evaluated for pyrosequencing and mid-IR analyses with at least $n = 17$ (maximum of 24) with all agro-ecosystems represented. Pyrosequencing data was analyzed according to the multiple sequence alignment performed using MUSCLE (with parameter – maxiters 1, -diags1 and -sv) (Edgar, 2004). Based on alignment, a distance matrix was constructed using DNAdist from PHYLIP version 3.6 with default parameters from Felsenstein (1989, 2005). These pairwise distances served as input to DOTUR (Schloss and Handelsman, 2005) for clustering the sequences into OTUs of defined sequence similarity that ranged from 0 to 20% dissimilarity. The OTU clusters were based upon a dissimilarity of 3% for generating predictive rarefaction models and for making calculations with richness (diversity) indexes (Chao and Bunge, 2002) in DOTUR.

Differences among microbial communities within fractions were characterized using UniFrac distances (Lozupone and Knight, 2005). UniFrac is a phylogenetic method that measures the distance between communities based on the lineages they contain. Non-metric multidimensional scaling (NMDS) then was used to illustrate UniFrac distances, and it also was used to illustrate the mid-IR spectral data. NMDS is an iterative ordination method that frequently is used for species composition data (Peck, 2010). It allows a choice of distance measure and conserves the rank order of sample dissimilarities in the rank order of NMDS distances. NMDS was conducted using the PC-ORD software package (MjM Software, Gleneden Beach, OR) in autopilot mode. The Sorensen distance measure was used for mid-IR data after square-root transformation. The final stress for each ordination solution (three-dimensional for UniFrac and two-dimensional for mid-IR) was 11.3 and 5.4, respectively, which is considered satisfactory (McCune and Grace, 2002).

Finally, the multiple response permutation procedure (MRPP) was used to determine whether aggregate-size treatments significantly affected bacterial community composition and chemical characterization (via mid-IR spectra). In MRPP, the test statistic A ranges from 0 to 1 and a higher A value indicates within-group homogeneity while the P value explains the possibility of the observed value due to chance (McCune and Grace, 2002).

3. Results

Pyrosequencing yielded a total of 294,913 sequences with an average of 3631 sequences, 1689 unique operational taxonomic units (OTUs) per sample (OTUs defined as having sequences that are ≥97% similar), and an average read length of 460 bp. This soil was associated with 25 phylum-level groups, and, on average, were dominated by the following nine phyla: *Proteobacteria* (32%), *Actinobacteria* (34%), *Bacteroidetes* (7%), *Chloroflexi* (8%), *Gemmatimonadetes* (4%), *Firmicutes* (4%), WS3 (3%), *Acidobacteria* (2%) and *Verrucomicrobia* (2%) (Table 2). Because of the great diversity of forms and functionalities found in *Proteobacteria*, this phylum was further subdivided into α-, β-, γ-, and δ-*Proteobacteria* that comprised 13, 5, 6, and 7% of all sequences, respectively. For the same reason, order *Rubrobacteriales* (16%) was independently interpreted from the total *Actinobacteria* phylum. Phyla that were less abundant but found in most samples included: OP10 (1%), *Planctomycetes* (0.9%), *Nitrospira* (0.8%), and BRC1 (0.5%) (Table 2).

3.1. Soil bacterial communities in whole soil and aggregate-size fractions

Each of the three soil aggregate-size fractions studied (MICRO, MACRO and SI + CL) supported distinct bacterial assemblages that were different from those found in WHOLE soil samples (Table 3 and Fig. 1). Using a cut-off R^2 value of 0.3, eleven phyla/sub-phyla had strong linear associations with the two ordination axes. The relative abundance of *Actinobacteria* (excluding order *Rubrobacteriales*) was strongly correlated with axis 1, indicating an association with the MACRO samples whereas *Nitrospira*, *Gemmatimonadetes*, OP10, WS3, and *Acidobacteria* showed a negative correlation with axis 1, indicating that were associated with SI + CL (e.g., had higher relative abundances). The relative abundance of order *Rubrobacteriales* was positively correlated with axis 2, indicating a strong association with MICRO samples. This group accounted for 20.5% in MICRO compared to an average of 14.6% in all other fractions plus WHOLE soil (Table 2). In contrast, relative abundances of *Bacteroidetes*, δ- and β-*Proteobacteria*, and *Verrucomicrobia* were negatively correlated with axis 2, indicating weak association with MICRO but strong associations with SI + CL and

Table 2
Mean relative abundance (%) for soil bacteria found in aggregate-size fractions and whole soil. Standard error is in parentheses.

Bacteria	Aggregate-size fractions			WHOLE soil
	MACRO	MICRO	SI + CL	
α -*Proteobacteria*	13.43 (0.41)	11.84 (0.48)	12.82 (0.48)	13.38 (0.57)
δ -*Proteobacteria*	7.85 (0.41)	7.13 (0.49)	9.03 (0.53)	7.38 (0.27)
γ -*Proteobacteria*	5.73 (0.37)	3.89 (0.39)	4.88 (0.33)	5.51 (0.54)
β -*Proteobacteria*	5.34 (0.31)	4.73 (0.26)	6.42 (0.26)	5.40 (0.24)
Rubrobacteriales	14.95 (0.90)	20.48 (1.58)	13.09 (0.83)	15.65 (0.72)
Actinobacteria[a]	19.96 (0.66)	19.04 (0.72)	15.15 (0.78)	18.09 (0.64)
Bacteriodetes	8.10 (0.45)	5.53 (0.62)	7.60 (0.39)	6.97 (0.31)
Chloroflexi	7.61 (0.33)	8.81 (0.50)	7.01 (0.21)	8.41 (0.38)
Gemmatimonadetes	4.11 (0.24)	4.76 (0.37)	5.91 (0.47)	4.38 (0.34)
Firmicutes	3.53 (0.16)	4.33 (0.37)	5.06 (0.21)	4.05 (0.17)
WS3	2.62 (0.15)	2.89 (0.28)	3.63 (0.23)	3.03 (0.15)
Acidobacteria	1.66 (0.09)	1.70 (0.15)	2.92 (0.24)	1.96 (0.11)
Verrucomicrobia	1.42 (0.09)	1.29 (0.10)	1.80 (0.13)	1.50 (0.08)
OP10	0.81 (0.06)	0.82 (0.07)	1.28 (0.09)	1.01 (0.05)
Planctomycetes	0.74 (0.06)	0.66 (0.06)	0.94 (0.06)	0.90 (0.06)
Nitrospirae	0.71 (0.07)	0.88 (0.10)	0.89 (0.10)	0.81 (0.08)
BRC1	0.43 (0.03)	0.52 (0.05)	0.55 (0.04)	0.53 (0.03)
TM7	0.44 (0.06)	0.16 (0.04)	0.27 (0.04)	0.37 (0.04)
Dictyoglomi	0.14 (0.02)	0.17 (0.03)	0.31 (0.04)	0.24 (0.03)
Candidatus Poribacteria	0.16 (0.02)	0.18 (0.02)	0.15 (0.02)	0.15 (0.02)
Bacteria incertae sedis	0.10 (0.02)	0.10 (0.02)	0.09 (0.02)	0.08 (0.01)
Chlamydiae	0.05 (0.01)	0.03 (0.01)	0.04 (0.01)	0.04 (0.01)
OD1	0.03 (0.01)	0.01 (0.01)	0.06 (0.02)	0.03 (0.01)
Chlorobi	0.02 (0.00)	0.01 (0.00)	0.03 (0.01)	0.03 (0.01)
Deferribacteres	0.01 (0.00)	0.02 (0.01)	0.03 (0.01)	0.03 (0.01)
Tenericutes	0.02 (0.01)	0.02 (0.01)	0.01 (0.00)	0.02 (0.01)
Deinococcus-Thermus	0.01 (0.003)	0.01 (0.003)	0.01 (0.005)	0.02 (0.007)
Spirochaetes	0.006 (0.004)	0.004 (0.004)	0.003 (0.002)	0.002 (0.002)
OP11	0.006 (0.003)	0.002 (0.002)	0.003 (0.002)	0.004 (0.002)
Thermodesulfobacteria	0.000 (0.000)	0.000 (0.000)	0.002 (0.002)	0.002 (0.002)

[a] *Actinobacteria* exclusive of *Rubrobacteriales*.

Table 3
Multi-response permutation procedure results for pairwise comparison for group differences between soil aggregates and whole soil.

Comparison	A[a]	p-value
WHOLE vs. MACRO	0.039	0.029
WHOLE vs. MICRO	0.068	0.004
WHOLE vs. SI + CL	0.153	<0.0001
MACRO vs. MICRO	0.096	0.001
MACRO vs. SI + CL	0.160	<0.0001
MICRO vs. SI + CL	0.127	0.0001

[a] A = chance-corrected within group agreement. $A < 0$ with more heterogeneity within groups than expected by chance. $A = 0$ when heterogeneity within groups equals expectation by chance.

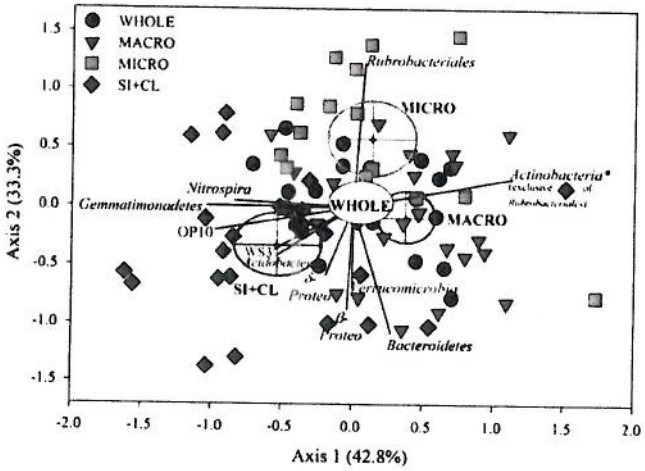

Fig. 1. Non-metric multi-dimensional scaling (NMDS) ordination plot derived from weighted pairwise UniFrac distances from WHOLE soil, MACRO (macroaggregates >250 μm), MICRO (microaggregates 53–250 μm) and SI + CL (silt + clay fraction; <53 μm). Individual points represent aggregate and whole soil samples and the ellipses represent the bidirectional 95% confidence interval of mean soil group scores. Vectors in the bi-plot overlay were constructed from a matrix containing relative abundances of each microbial phyla/subphyla as well as SOC and TN contents. Only correlations >0.3 were included. The angle and length of the vector indicate the direction and strength of the variable and the NMDS axis. Abbreviations: δ-*Proteo* = δ-*Proteobacteria*; β-*Proteo* = β-*Proteobacteria*.

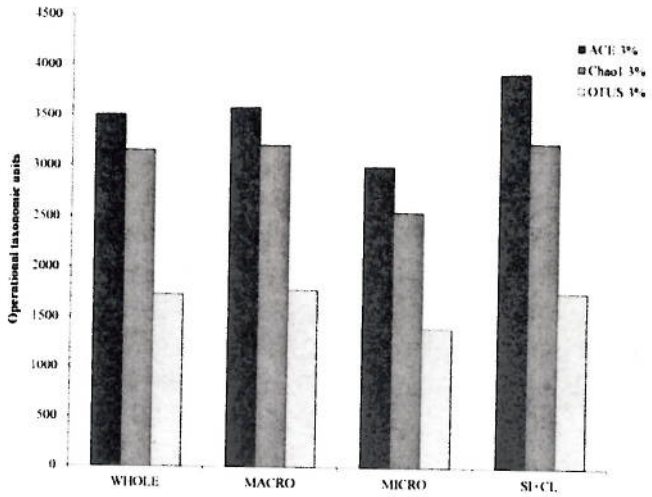

Fig. 2. Bacterial richness indices of aggregates and whole soil at a genetic distance of 3%, expressed as the number of observed unique operational taxonomic units (OTUs), abundance-based coverage estimator (ACE) and Chao1.

MACRO. The relative abundances of many bacterial phyla in the WHOLE soil samples more closely follow those associated with MACRO than the other two aggregate fractions (SI + CL or MICRO) (Table 2). This is also evident in the NMDS plot where the two ellipses overlap between MACRO and WHOLE. Although the MRPP analysis calculated a p-value <0.05, this does not account for error associated with multiple comparisons. Using a Bonferroni adjustment, our calculated p-value of 0.03 for the comparison between WHOLE and MACRO would not be statistically significant. All other pair-wise comparisons, however, remained significant with this adjustment. The first eight phyla/subphyla listed in Table 2 constitute at least 76% of the community. The greatest relative abundance of four of these groups (α- and γ-*Proteobacteria*, *Actinobacteria* excluding order *Rubrobacteriales*, and *Bacteroidetes*) was found in the MACRO fraction. The MICRO fraction had the greatest relative abundances of only *Rubrobacteriales* and *Chloroflexi* and SI + CL had the greatest relative abundances of δ- and β-*Proteobacteria*. In contrast, the next nine phyla, which represented the more rare groups (i.e., those which accounted for 6% or less of the total community), were associated with SI + CL.

Equally high diversity levels (according to rarefaction curves at 3% dissimilarity) were found between MACRO and SI + CL fractions (1781 and 1765, respectively) (Fig. 2). The lowest level using this index was found for the MICRO fraction (1406). A similar trend was shown by the Shannon index. Although the SI + CL fraction had the greatest diversity measures according to ACE and Chao1 indexes (3934 and 3247, respectively), the MICRO fraction had the lowest diversity measures, regardless of the index used (Fig. 2).

3.2. Soil organic C and chemical composition of soil using mid-IR spectroscopy

Among soil aggregate fractions, the MACRO fraction contained the greatest quantity of C (4.9 mg C g^{-1} aggregate) and N (0.36 mg N g^{-1} aggregate) followed by SI + CL (3.0 mg C g^{-1} aggregate and 0.27 mg N g^{-1} aggregate) and MICRO (2.8 mg C g^{-1} aggregate and 0.2 mg N g^{-1} aggregate) (data not shown). The WHOLE soil and aggregate fractions differed in their mid-IR spectra and, thus, their mineral and organic chemical composition (Fig. 3A; MRPP, A value > 0.06, p-value < 0.005 for all comparisons). The WHOLE soil, with higher scores along axis 1, had higher absorbance in the 1750–1550 cm^{-1} region (Fig. 3B), which has bands associated with several organic features such as carboxylic acids, amide bonds, aromatics, methyl groups and phenolics among others (Baes and Bloom, 1989; Janik et al., 2007; Calderón et al., 2011b). The WHOLE soil also showed high correlation with the wide region between 3630 and 2830 cm^{-1}, which includes bands for clays, OH/NH bonds, and aliphatic CH bonds (Nguyen et al., 1991). The MICRO fraction was positively correlated with axis 2 in the 1780–2000 cm^{-1} region (Fig. 3A, B), indicating silicate bands (Nguyen et al., 1991). The SI + CL fraction and WHOLE soil correlated with absorbance at 1290–1080 cm^{-1}, which includes bands for resistant aromatics and labile fulvic acids, as well as clays (Janik et al., 2007; García-Gil et al., 2008).

3.3. Coupling of pyrosequencing data with mid-IR spectra at the aggregate scale

Different bacterial phyla were associated with particular soil chemistries, and these relationships were unique for each soil aggregate fraction (Fig. 4 A–E). The MACRO fraction was characterized by positive correlations between the relative abundances of *Bacteroidetes* and *Verrucomicrobia* with the entire spectra whereas *Acidobacteria* was correlated negatively (Fig. 4A). The MACRO fraction was also characterized by a positive correlation ($r > 0.4$) between δ-*Proteobacteria* and a negative correlation ($r < -0.2$) between α-*Proteobacteria* with the 1370–1450 cm^{-1} spectra region, corresponding to CH_2, CH_3, COO, COOH aliphatics and phenolics (Janik et al., 2007). These same bacterial groups displayed different and more variable responses across the spectra in MICRO (Fig. 4B, C) and SI + CL (Fig. 4D, E).

The MICRO fraction was associated with the greatest number (10) of phyla/subphyla with strong correlations across the spectra and resulted in two groups with opposing patterns (Fig. 4B, C). For example, the patterns associated with *Actinobacteria* (excluding order *Rubrobacteriales*) are opposite to those found for members belonging to the order *Rubrobacteriales*. The 1370–1450 cm^{-1} region illustrated a large shift in *Rubrobacteriales* from a negative correlation (−0.3) to a positive one (0.3), whereas *Actinobacteria*

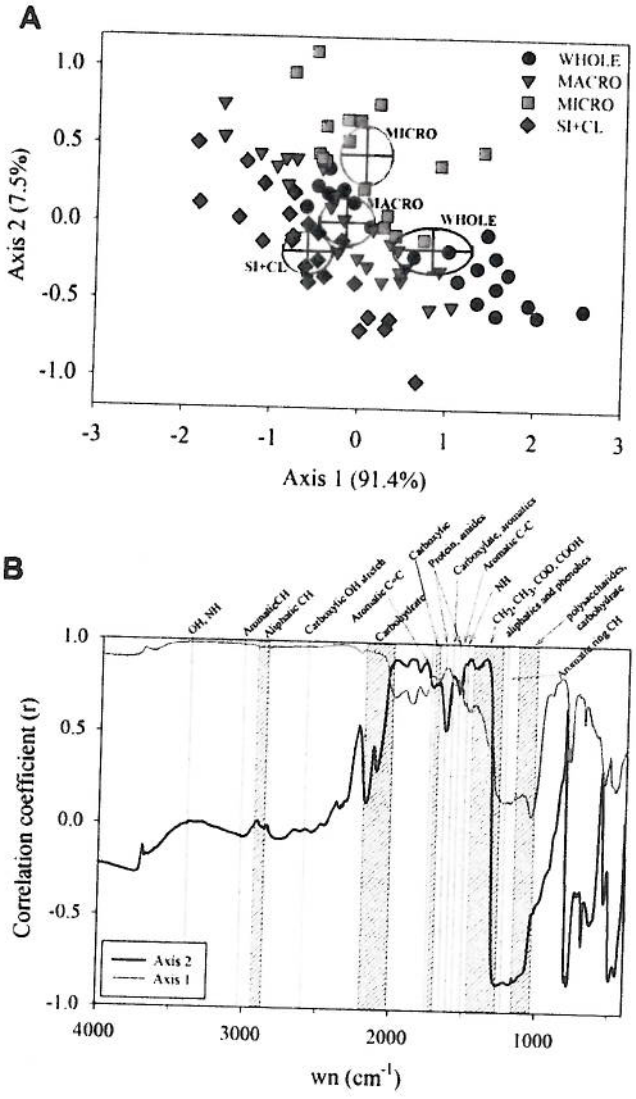

Fig. 3. (A) Non-metric multi-dimensional scaling (NMDS) ordination plot derived from absorbance readings at each wavelength within the mid-IR spectrum from WHOLE soil, MACRO (macroaggregates >250 μm), MICRO (microaggregates 53–250 μm) and SI + CL (silt + clay fraction; <53 μm). Data were square-root transformed. Individual points represent aggregate and whole soil samples and the ellipses represent the bidirectional 95% confidence interval of mean soil group scores. (B) Correlation coefficients between scores along each axis and absorbance readings across the spectrum for the NMDS ordination plot shown in 3A.

abruptly showed a shift from 0.8 to −0.5. The different classes of *Proteobacteria* also showed distinct patterns across the 1370–1450 cm^{-1} region, which represents CH_2, CH_3, COO, COOH aliphatics and phenolics. Correlations of β-, γ-, and δ-*Proteobacteria* abruptly shifted from strongly positive values to strongly negative values as α-*Proteobacteria* correlation coefficients shift in the opposite manner.

The SI + CL fraction was characterized by a negative correlation between δ-*Proteobacteria* with the 2200–4000 cm^{-1} region and a positive correlation with the 2000 cm^{-1} band, typically associated with carbohydrate −COH (Fig. 4E). Opposing correlation patterns across the spectra between order *Rubrobacteriales* and *Actinobacteria* phyla, as seen in the MICRO fraction, are also evident in SI + CL (Fig. 4D, E). *Acidobacteria* showed positive correlations with the 1370–1450 cm^{-1} and 1500 cm^{-1} regions (Fig. 4D).

Although each aggregate-size fraction showed distinct bacterial groups associated with specific soil chemistries, MACRO and SI + CL fractions had negative correlations between *Gemmatimonadetes* and *Chloroflexi* phyla and various properties located between 2000 and 4000 cm^{-1} and this pattern was opposite for *Bacteroidetes* which were positively associated with the same area of the spectra. Additionally, MICRO and SI + CL fractions both revealed opposing correlation patterns between *Gemmatimonadetes* and *Bacteroidetes* within the 2200 and 4000 cm^{-1} and the 1370–1450 cm^{-1} regions. *Chloroflexi* and *Firmicutes* exhibited similar correlation patterns as *Gemmatimonadetes* (data not shown). *Acidobacteria* was primarily negatively correlated across the majority of the regions of the spectra in MICRO fractions, except for a strong positive correlation found with the 1370–1450 cm^{-1} region, which includes bands for C–H bending; aromatic C=C, C=N in amides; and $CO–CH_3$ (Calderón et al., 2011b).

4. Discussion

4.1. Bacterial communities in whole soil and soil microenvironments

Our results agree with previous studies in that different aggregate-size classes (MACRO, MICRO, and SI + CL) support distinct microbial habitats compared to the WHOLE (bulk) soil typically sampled (Sessitsch et al., 2001; Mummey and Stahl, 2004; Mummey et al., 2006). Our study, however, provided unique information that related bacterial assemblage composition to chemical composition of SOM within these microenvironments. Although our results provide a more robust assessment of bacterial distribution and diversity within aggregates by using pyrosequencing than explored before, DNA may persist in soil (Greaves and Wilson, 1969), via protection by complex interactions between soil minerals (sand and clay), humic substances, and organo-mineral complexes, possibly resulting in amplification of extracellular bacterial DNA.

Two microbial groups, the *Rubrobacteriales* and the *Acidobacteria*, had relative abundances that were different from previously published work. In general, *Rubrobacteria*, comprised up to 21% of total bacterial abundance in our soil with greater proportions in the MICRO fraction. Although this taxon has not been extensively studied, some research has shown members of *Rubrobacteria* to be tolerant to gamma-radiation (Yoshinaka et al., 1973) and desiccation (Singleton et al., 2003). These findings may explain their prevalence in our soil which experience high soil temperatures and long, dry periods during summer months. Similar to our findings, Mummey et al. (2006) reported high abundance of *Rubrobacteriales* in the protected inner-aggregate microaggregates and suggested that they may play an important role in microaggregate reformation. In our soil, low C content coupled with the strong presence of *Rubrobacteriales* in the 'unprotected' MICRO fraction (i.e., we did not measure inner-aggregate microaggregates) supports the concept proposed by Mummey et al. (2006) that these microaggregates have recently been formed. Because of the predominance of *Rubrobacteriales* in the MICRO fraction and the proposed importance of this fraction in long-term C sequestration (Skjemstad et al., 1990; Six et al., 2000), more research is needed on this taxon independent from the *Actinobacteria* phyla to determine the role of this taxon in soil C storage.

In our study, *Acidobacteria*, regardless of location within the soil microenvironment, showed an order of magnitude lower (~2%) than reported in other studies (~20%); even in soils with neutral to slightly alkaline pH (Janssen, 2006; Lauber et al., 2009). Our lower values are, however, similar to those found in a study of grass pastures in Scotland (McCaig et al., 1999) and other semiarid soils

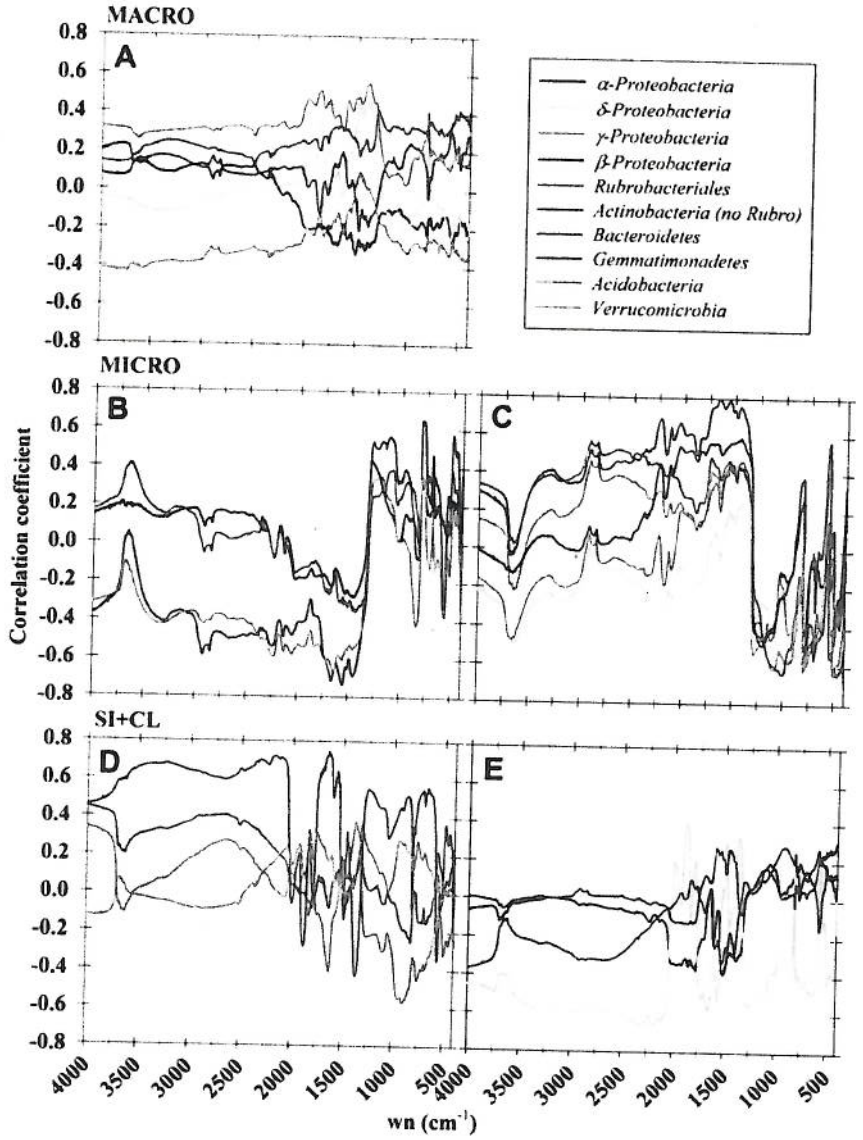

Fig. 4. Correlation coefficients across the mid-infrared spectrum between the relative abundance of several bacterial phyla/subphyla and absorbance at each mid-IR wavelength for MACRO (A), MICRO (B and C), and SI + CL (D and E) fractions. Only phyla that had particularly high positive or negative r scores ($-0.2 < r > 0.2$) were included.

(Acosta-Martinez et al., 2008). Our results agree in part with Sessitsch et al. (2001) who reported that the *Acidobacterium* group mainly colonized silt and clay fractions. Mummey et al. (2006) reported greater relative abundance of *Acidobacteria* in the MICRO fraction, while we found this group of bacteria was equally abundant in MACRO and MICRO fractions. Mummey et al. (2006), however, did not specifically investigate the SI + CL fraction. Although predominance of *Acidobacteria* has been associated with low C soils (Fierer et al., 2007), we found that C quantity differences between aggregates may not solely explain *Acidobacteria* predominance. Both MICRO and SI + CL fractions contained equally low C content compared to the MACRO fraction, but the SI + CL fraction contained 71% greater relative abundance of *Acidobacteria* than the MICRO fraction.

Bacterial diversity indexes were not sensitive measures to distinguish soil aggregate fractions. In contrast to other studies where greater bacterial diversity was observed in small size fractions (typically <53 μm) than in larger size particles (Sessitsch et al., 2001; Kirchmann et al., 2005), we found equally high diversity levels in MACRO and SI + CL fractions than the MICRO fraction. This discrepancy may be related to the method used to isolate aggregates. Our method was based on that used by Elliott (1986), which extracts water-stable aggregates and does not extract protected smaller aggregates and mineral fractions existing within macroaggregates as described by Six et al. (1998). It is possible that the greater disruptive forces such as those used in the studies by Sessitsch et al. (2001) and Kirchmann et al. (2005), resulted in a greater proportion of silt and clay extracted when compared with our method. The lower bacterial diversity in MICRO for our study may be due in part to dominance by soil fungi as proposed by Kong et al. (2011). Perhaps the bacterial diversity indexes may not be different whereas fungal diversity indexes could have reflected more distinct values, but the fungal component was not addressed in our study. Other studies have also reported that diversity indexes are not necessarily useful in predicting or understanding important soil processes such as C sequestration (King, 2010; Lauber et al., 2009). Furthermore, Cleveland and Liptzin (2007) proposed that variability within

specific microbial assemblages likely impact C storage irrespective of overall diversity. It may be that the distributions of the more rare bacterial phyla (e.g., those representing less than 6% of the identified population) may be more important for differentiating communities in soil aggregate-size fractions, than measures of overall bacterial diversity (e.g., richness estimates).

4.2. Mid-IR analysis in whole soil and soil microenvironments

Physical fractionation of soil microenvironments showed clear differences on their spectroscopic properties with a predominant effect from mineral features. All three fractions lost absorbance at 3400 cm^{-1} relative to WHOLE soil, but the effect was more pronounced on the SI + CL fraction. The 3400 cm^{-1} band is important because this OH or NH stretching feature marks presence of relatively labile SOM (Haberhauer and Gerzabek, 1999; Calderón et al., 2011b). It is possible that this band was lost with soluble SOM during fractionation, but it also suggests that the MICRO and MACRO fractions contained overall older, more processed C relative to WHOLE soil. The band at 2930–2870 cm^{-1} indicated aliphatic CH stretching of methyl and methylene groups, which marks C in the soil light fraction (Calderón et al., 2011b) and is also characteristic of high quality prairie soils (Calderón et al., 2011a). Spectral subtraction (not shown) indicated that absorbance in this region increased slightly in MICRO and MACRO during fractionation, but declined in the SI + CL fraction. However, this band was not pronounced in WHOLE soil to begin with, which may have diminished potential effects of fractionation. The MICRO fraction had more absorbance at 2000–1750 cm^{-1} and at 1344 cm^{-1}, indicating strong influence of silicates and, thus, sand enrichment compared to other fractions. Alternatively, MICRO spectra may have more silicate signal because of decreased coating of SOM or other interfering substances. Spectral subtraction also indicated that the MICRO fraction does absorb more than the WHOLE soil in the region between 1600 and 1350 cm^{-1} suggesting that a variety of resistant and labile organic functional groups like aromatics, phenolics and proteins are enriched in MICRO.

Differences between MACRO and MICRO fractions are also dominated by increased silicate signal in the MICRO fraction. Nevertheless, the organic makeup of the MICRO fraction does differ as indicated by higher absorbance at the carboxylic band at 1700–1740 $cm^{-1}$and bands between 1530 and 1450 cm^{-1}. It is possible that increased absorbance of MICRO at these frequencies indicated that they are older and more stable forms of C. Research as to the resistant or labile character of many of these narrow bands is ongoing and future studies will help elucidate their role in soil C sequestration. Caution is necessary when assigning spectral features to specific chemical characteristics in soil using mid-IR techniques. Peak identification is not exact because spectral bands occur across a wide range and there is some degree of overlap between bands. Nevertheless, studies such as this, relating biological attributes to spectral data will help us build a knowledge base and shed light on the meaning of specific mid-IR absorbance in soil.

4.3. Coupling pyrosequencing data with mid-IR at the aggregate scale

Our mid-IR results suggested that the relative abundances of soil bacteria within soil aggregate fractions are driven more by shifts in chemical composition of SOM than SOC quantity. The impact of changes in SOM chemistry on the bacterial composition was specific to each aggregate. However, it is equally possible that these SOM compounds may be inaccessible and not directly influence the community composition. Regardless, our data suggest that the effect of changes in SOM chemistry on the bacterial composition may be defined by aggregate specific patterns. For example, within each aggregate-size class, the most prevalent bacterial groups were not those with strong relationships across the mid-IR spectra and suggest that low abundant bacterial groups responded more dynamically to shifts in SOM composition than the most dominant groups. The distribution of bacterial populations within and among these microenvironments, as well as the chemical composition of SOM is partially driven by feedback loops between the activities of soil microorganisms, which alter SOM chemistry and thus their environment, and the original composition of the organic matter itself. As microbial communities often work as a consortia to degrade complex organic materials such as those found in soil, it is probable that dominant groups benefit from co-metabolites produced by less dominant groups. When C is plentiful and sources include relatively degradable components, such as in the MACRO fraction wherefresh residues would be expected, few groups responded to shifts in C. In contrast, the MICRO fraction, which contained the lowest amount of C and had the lowest diversity indexes, had the most bacterial groups with strong associations with specific SOM chemistries. *Rubrobacteriales* was not one of the groups strongly correlated along the spectra, but was the most dominant within the MICRO fraction. It is uncertain whether a lack of correlation between relative abundances of bacterial phyla with various SOM chemistries suggests that these groups are less important to processing these compounds or whether dominant groups do not need to be sensitive to minor changes in SOM chemistries. In other words, less abundant groups need to compete more strongly to survive whereas dominant groups benefit from sheer numbers and the co-metabolites produced from the entire microbial consortia.

Within a given aggregate fraction such as MICRO, opposing patterns between *Rubrobacteriales*, *α-Proteobacteria*, *Gemmatimonadetes*, and *Acidobacteria* taxa and *Bacteriodetes*, γ- and β-*Proteobacteria* and *Actinobacteria* (excluding *Rubrobacteriales*) suggest that these groups occupy different ecological niches. The opposing patterns between order *Rubrobacteriales* and *Actinobacteria* phyla, seen in MICRO and SI + CL fractions was unexpected but supports research reported by Bryan and Johnson (2008), which suggested that *Rubrobacteriales* may have dissimilatory physiological characteristics compared to other members of *Actinobacteria*. We also found that the relative abundance of *Rubrobacteriales* was high when the relative abundance of *Proteobacteria* was low ($r = 0.85$), suggesting that these groups directly compete with each other for the same ecological niche. This relationship was also observed in the Scottish grassland soils where *α-Proteobacteria* was more prevalent and *Rubrobacteriales* was less abundant in improved grasslands (McCaig et al., 1999).

Factors other than the chemical composition of soil, such as moisture and oxygen gradients, likely influence soil bacterial distribution and diversity (Hansel et al., 2008). Although the phylum *Verrucomicrobia* is known to be ubiquitous in most soils (Zhang and Xu, 2008), it is probable that this group is generally less common in arid soils due to their sensitivity to dryness (Sessitsch et al., 2001). Members of this phylum were most prevalent in SI + CL fractions, which is where higher moisture content is most likely to exist. However, members from this phylum have been found to utilize a narrow range of carbohydrates (Sessitsch et al., 2001; Hedlund et al., 1996) which may explain why they tended to have strong positive correlations with only narrow regions along the mid-IR spectra in MACRO and MICRO fractions.

5. Conclusion

Integration of pyrosequencing, mid-IR spectroscopy, and soil fractionation techniques allowed us to discern relationships

between bacterial assemblages and soil chemical composition within aggregates at a level previously not achieved. Our study suggests that the relative abundance of soil bacteria within soil aggregate fractions are driven more by shifts in chemical composition of SOM than C quantity (content). Specific bacterial assemblages were associated with particular soil chemistries within the soil microenvironment and these associations differed from those found in the whole (non-fractionated) soil samples. The less dominant bacterial taxa were more important for differentiating between communities in soil microenvironments and revealed the importance of robust molecular tools in soil ecological studies. Our findings that the less dominant bacterial taxa showed stronger relationships with specific chemical functional groups than the more dominant bacterial taxa should be further explored to reveal potential linkages between specific bacterial assemblages with C sequestration. Our approach can serve as a platform for future studies focused on exploring the complex relationships involving soil biodiversity and C distribution at the aggregate scale.

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