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The characterization and composition of bacterial communities in soils blended with spent foundry sand

Robert S. DUNGAN[1]*, Jong-Shik KIM[2], Hang-Yeon WEON[3], April B. LEYTEM[1]

[1]USDA-Agricultural Research Service, Northwest Irrigation and Soils Research Laboratory, 3793 North 3600 East, Kimberly, Idaho 83341, USA
[2]Gyeongbuk Institute for Marine Bioindustry (GIMB), 688-3 Hoojung-Ri, Jukbyeon-Myeon, Uljin-Gun, Gyeongsangbuk-Do 767-813, Republic of Korea
[3]Korean Agricultural Collection, National Academy of Agricultural Science, Rural Development Administration, Seodung-dong 88-20, Suwon 441-853, Republic of Korea

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ABSTRACT – The purpose of this research was to characterize the structure and composition of bacterial communities in sandy loam and silty clay soils amended with 30% spent sand from iron, aluminum, and steel foundries. All spent foundry sand (SFS) blends were grown with or without perennial ryegrass and samples were collected at 4 weeks and 6 months. Regions of the 16S rRNA gene were amplified using PCR and subsequently analyzed by DGGE and sequenced for bacterial identification and phylogenetic classification. Cluster analyses of PCR-DGGE banding patterns revealed that SFS blends from week 4 and month 6 produced unique clusters, with most ryegrass treatments clustering away from those without ryegrass. The diversity of the bacterial community revealed that it was generally higher in the SFS blends without ryegrass. By month 6 in treatments without ryegrass, the diversity in the sandy loam blends was similar to the control, while the diversity in all silty clay blends was greater than the control. Phylogenetic analysis of bacterial isolates (total of 309) from the SFS blends showed that they were dominated by Actinobacteria (46%), Proteobacteria (29%), and Bacilli (20%), with fewer numbers belonging to Bacteroidetes (5%). While the addition of SFS to soil does bring about bacterial community level changes, these changes are similar to that of blending soil with clean silica sand.

Key words: bacterial communities; PCR-DGGE; phylogeny; spent foundry sand; 16S rRNA gene.

INTRODUCTION

The foundry industry uses millions of tons of sand each year to create metalcasting molds and cores. Much of this sand is reclaimed within the foundries, but it is eventually discarded after a reduction in grain size and shape makes the sand unsuitable for continued use. In the United States, approximately 13 millions tons of SFS are generated each year (U.S. EPA, 2002). Over the last several years, interest in using SFSs as aggregate in manufactured soils (e.g. topsoils, potting soils, landscaping mixes) and geotechnical applications has come to the forefront. The U.S. EPA (2007) recently released a statement endorsing the beneficial use of spent sands from ferrous and aluminum foundries in agricultural, horticultural, and geotechnical applications. The beneficial use of industrial byproducts, like SFS, preserves natural resources by decreasing the demand for virgin materials, conserves energy, and reduces greenhouse gas emissions through reduced mining activities, and decreases the economic and environmental burdens of disposal.

* Corresponding Author. Phone: +1 208 423 6553; Fax: +1 208 423 6555; E-mail: robert.dungan@ars.usda.gov

Only several states allow SFSs to be beneficially used in manufactured soils (U.S. EPA, 2002), but their use in additional states has been limited due to the perception that they contain elevated concentrations of trace metals and xenobiotics. Dungan and Dees (2009) have shown that iron, steel, and aluminum SFSs contain trace metals at concentrations below those found in most native soils. Xenobiotics, such as polycyclic aromatic hydrocarbons, phenolics, and dioxins, were also found at very low concentrations in a variety of SFSs (Dungan, 2006; Dungan *et al.*, 2009). Because of the low trace metal and xenobiotic concentrations in most SFSs, they are unlikely to present a significant risk to environmental receptors when used in soil-related applications. In a study conducted by Dungan and Dees (2006), artificial soil blended with up to 50% SFS (dry wt) did not affect earthworm viability, except in blends containing a brass SFS with high concentrations of copper, lead, and zinc. Similarly, the dehydrogenase activity (DHA; a general indicator of microbial activity), was significantly reduced in a sandy loam soil when blended with 10, 30, and 50% brass SFS (Dungan *et al.*, 2006). Compared to soil blended with plain silica sand, impacts on the DHA in iron and aluminum SFS blends were absent during the 12-week study.

TABLE 1 - Metal concentrations in the Matawan soil, Blount soil, silica sand, and spent foundry sands (mg kg^{-1})

	Al	As	Ba	Cd	Co	Cr	Cu	Fe	Mg	Mn	Mo	Ni	Pb	Sb	Se	Te	Tl	V	Zn
Matawan sandy loam	7097	2.8	35.1	<0.03	2.4	10.6	9.3	6894	894	108	<0.1	4.4	42.4	0.4	<1.1	<0.5	<0.5	14.9	26.4
Blount silty clay	30123	6.5	116	<0.03	11.6	37.0	14.6	28716	5170	446	2.8	23.0	18.3	2.2	<1.1	<0.5	<0.5	70.8	55.7
Silica sand	76.5	<0.9	<0.01	<0.03	<0.02	<1.9	1.1	73.3	7.5	0.3	<0.1	<0.04	0.2	<0.2	<1.1	<0.5	<0.5	<0.02	2.0
IGS-1	4319	0.9	10.8	<0.03	0.1	1.9	1.2	2327	976	35.2	0.1	0.7	3.3	<0.2	<1.1	<0.5	<0.5	2.6	13.3
IGS-2	5508	0.9	22.0	<0.03	0.1	2.1	1.2	2782	1414	23.7	0.1	1.0	4.1	<0.2	<1.1	<0.5	<0.5	2.7	8.8
AGS-1	5541	<0.9	6.5	<0.03	0.1	3.1	18.4	2734	1285	36.4	<0.1	1.0	2.1	<0.2	<1.1	<0.5	<0.5	2.6	13.1
AGS-2	11957	<0.9	19.0	<0.03	0.7	4.8	12.6	3796	2183	188	0.7	7.2	1.9	<0.2	<1.1	<0.5	<0.5	5.1	33.0
SPU	349	<0.9	1.4	<0.03	<0.02	<1.9	2.8	7204	79.6	13.3	<0.1	0.8	1.6	0.3	<1.1	<0.5	<0.5	2.3	12.9

Bacteria are important organisms in soils and are involved in essential processes such as cycling of nutrients, formation of humus, stabilization of soil structure, and degradation of organic pollutants. Inputs of toxic elements can alter the biological activity of soil microorganisms, sometimes causing a severe ecosystem disturbance. Affected soils often exhibit decreased microbial diversity, microbial biomass and enzyme activities, and lower respiration rates per unit biomass. An increasing body of evidence suggests that microorganisms are more sensitive to heavy metal pollution than the faunal or floral community growing on the same soil (Giller *et al.*, 1999). While SFSs contain low trace metal and xenobiotic concentrations, no studies have been conducted to assess their influence upon the soil bacterial communities.

The objectives of this laboratory study were to utilize molecular-based methods to characterize the structure and composition of bacterial communities in soils that have been blended with iron, aluminum, or steel SFSs. The treatments were grown with and without perennial ryegrass to assess the effect of the root zone on the bacterial community. Differences in the bacterial community structure were determined via polymerase chain reaction (PCR) amplification of 16S ribosomal DNA (rDNA) followed by denaturing gradient gel electrophoresis (DGGE). A PCR-amplified region of 16S rDNA from bacterial isolates was sequenced and subsequently used for identification and phylogenetic classification.

MATERIALS AND METHODS

Soils and foundry sands. The soils used in this study were Matawan sandy loam (fine-loamy, siliceous, semiactive, mesic Aquic Hapludults) and Blout silty clay (fine, illitic, mesic Aeric Epiaqualf). Both soils were collected from the Ap horizon of an agricultural field, passed through a 2-mm sieve, and stored at 5 °C prior to their use. The Matawan soil has a pH of 5.7, total carbon content of 1.0%, and a maximum water holding capacity (WHC_{max}) of 0.26 kg kg^{-1}. The Blount soil has a pH of 5.8, total carbon content of 3.5%, and a WHC_{max} of 0.25 kg kg^{-1}.

The silica sand was obtained from U.S. Silica (Berkeley, WV, USA) and is specifically graded for use as foundry molding sand. The silica sand was used as received. The SFSs were obtained from iron, aluminum, and steel foundries (Table 1). The iron and aluminum SFSs were green sands, where bentonite clay was used as the binder. The binder used in the steel SFS was a phenolic urethane resin. All SFSs were passed through a 2-mm sieve and thoroughly homogenized before being blended with the soils.

Experimental design. The soils were blended with 30% SFS or silica sand (dry wt) or left untreated and allowed to acclimate for 3 months at room temperature after the moisture content was adjusted to 50% of the WHC_{max}. Based on previous research, 30% SFS was chosen as a realistic maximum amount of sand that could be applied to soils or used in manufactured soils (Dungan *et al.*, 2007). After 3 months, 300 g (dry weight) of the soils or blends were placed in small plastic pots placed on saucers. Four replicates per treatment were planted with perennial ryegrass (*Lolium perenne* L.), while four additional replicates were left unplanted. The treatments were incubated in a growth chamber at 20 °C and 50% humidity, under a light-dark cycle of 16 h light and 8 h darkness, and maintained at 50% of the WHC_{max} with distilled water. Samples were aseptically collected from each replicate at week 4 and month 6 using ethanol-disinfected spatulas and immediately frozen at -20 °C until processed.

PCR-DGGE. Prior to DNA extraction, the replicates from each treatment were pooled. The bacterial community DNA was extracted using a FastDNA Spin Kit (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol. PCR amplification of the 16S rDNA was performed with the forward primer F352T [5'-ACTCCTACGGGTGGC-3'] and reverse primer 519r [5'-ACCGCGGCTGCTGGCAC-3'], which target the V3 region of Eubacteria (Crump *et al.*, 2003; Ahn *et al.*, 2006). A 40 nucleotide GC-clamp was attached to the 5' end of the F352T primer. The PCR reaction mixtures were prepared as previously described by Ahn *et al.* (2006). The PCR temperature conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were subjected to denaturing gel electrophoresis (DGGE) analysis with the Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) as previously described by Ferris *et al.* (1996). The PCR products were loaded onto 8% (w/v) polyacrylamide gels with a denaturing gradient ranging from 40 to 70% (100% denaturant contains 7 *M* urea and 40% formamide). The gels were electrophoresed for 17 h at 60 °C and a constant voltage of 40.

After electrophoresis, the gels were stained with SYBR Green I (Cambrex BioScience, Rockland, ME, USA) for 15 min, rinsed for 25 min, and photographed on a UV transilluminator. The digitized images were then imported into GelCompar II (Version 3.5, Applied Maths, Inc., Austin, TX, USA) for fingerprint processing. After the gel lanes were defined, a densitometric curve was calculated for each lane after background subtraction, then the lanes were normalized using two reference lanes on each gel. Similarity indices between densitometric curves were calculated by Pearson correlation (Hane *et al.*, 1993) and dendrograms were constructed using the unweighted pair group method using arithmetic averages (UPGMA). The determination of structural diversity according to the Shannon-Weaver index ($H = -\Sigma g P_i \log P_i$), where P_i is the ratio of peak height to the sum of all peak heights), was calculated on the basis of the number of DGGE bands and their relative intensities (Eichner *et al.*, 1999).

Sequence analysis of isolates. The bacterial isolates were obtained by serially diluting the soils and blends using a 0.85% saline solution, and then plating on DR2A agar (Tamaki *et al.*, 2005). After seven days of incubation at 28 °C, single colonies were purified by transferring them onto fresh DR2A plates.

The 16S rDNA from the isolates was amplified using the universal primers fD1[5'-AGAGTTTGATCCTGGCTCAG-3'] and rP2 [5'-ACGGCTACCTTGTTACGACTT-3'] (Weisburg *et al.*, 1991). Their partial nucleotide sequences were determined with an Applied Biosystems 3100 sequencer (Applied Biosystems, Foster City, CA, USA) by using the 510R sequencing primer [5'-TATTACCGCGGCTGCTGGCA-3'] and DNA sequencing kit (BigDye terminator Cycle Sequencing Ready Reactions v3.1; Applied Biosystems). 16S rDNA sequences were aligned using the NAST aligner, and the aligned sequences were classified using the Greengenes web site (DeSantis *et al.*, 2006a, 2006b). The phylogenetic tree was constructed by the neighbor-joining method with MEGA3 (Kumar *et al.*, 2004). Bootstrap analyses of the neighbor-joining data were used to assess the stability of the relationships. The 16S rDNA sequence data from the isolates were placed in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession numbers EU839048-EU839356.

RESULTS AND DISCUSSION

PCR-DGGE

In this study, two iron green sands (IGS), two aluminum green sands (AGS), and one steel phenolic urethane sand (SPU) were blended into sandy loam (Matawan) and silty clay (Blount) soils to assess their influence upon the bacterial community over a 6-month period. The data in Table 1 shows that the SFSs used in this study contain low trace metal concentrations. In fact, the trace metal concentrations in most cases are lower than in the Matawan and Blount soils. To analyze the structure of the bacterial community in the different SFS blends, DGGE patterns of the 16S rDNA fragments were analyzed using cluster analysis and the Shannon-Weaver index of diversity, *H*.

The results from the Matawan soil blends show that distinct clusters were produced at week 4 and month 6 (Fig. 1A). Furthermore, the blends containing perennial ryegrass generally clustered away from those blends without ryegrass. Grasses have been shown to influence the soil microbial community when grown in monoculture (Grayston *et al.*, 1998; Steer and Harris, 2000; Singh *et al.*, 2007). Although Singh *et al.* (2007) observed that microbial communities were influenced by plant growth, there was no evidence that different grass species selected for the soil microbial communities in the rhizosphere. When a single grass species was used, it was determined that the major determinant of community structure in the rhizosphere was soil type.

At week 4, three unique clusters were present, with DGGE pattern similarities ranging from 70 to 95%. In the month 6 samples, two unique clusters were present, with pattern similarities ranging from 40 to 91%. In general, the pattern of the bacterial community was most similar between those blends containing the SFS, while the soil only controls and silica sand blends (with and without ryegrass) were least similar to the SFS blends. In fact, the soil controls and silica sand blends were more similar to the SFS blends at week 4, than at month 6. Contrary to what we expected, this data suggests that the bacterial community in the SFS blends is shifting away from the natural background population with time.

In the Blount soil blends, the samples collected at week 4 and month 6 produced distinct clusters (Fig. 1B); however, only the soil control (with ryegrass) at month 6 clustered with the week 4 samples (57% similarity). As in the Matawan soil blends, the Blount soil blends containing perennial ryegrass also clustered away from those without ryegrass. It can certainly be hypothesized that the rhizosphere of the perennial ryegrass is supporting different bacterial populations than in blends without ryegrass. At week 4, DGGE pattern similarities within the clusters ranged from 73 to 97% and, at month 6, ranged from 68 to 91%. At week 4 and month 6, the soil only control and silica sand blends (with and without ryegrass) clustered tightly among the SFS blends. These results are in contrast to those obtained in the Matawan blends at month 6, indicating that the bacterial community in the Blount SFS blends are more similar to the controls.

The Shannon-Weaver diversity values, *H*, from the blends with and without ryegrass are shown in Table 2. Higher *H* values indicate increased diversity of the dominant bacterial community. In general, *H* was the highest in the blends without ryegrass at both week 4 and month 6, compared to those with ryegrass. At week 4, the highest *H* was present in the IGS-1 and IGS-2 blends (both soils without ryegrass). In the Matawan soil control, *H* was 1.29, with the lowest *H* of 1.18 in the SPU blend. In the Blount soil, *H* in the SPU blend was 1.43 versus 1.38 in the control. In the Matawan blends with ryegrass at week 4, most of the *H* values were slightly greater than that of the control, but in the Blount blends, all of the *H* values were less than the soil control. At month 6, *H* values in Matawan blends without ryegrass were similar to the control, except in the IGS-1 and AGS-1 blends, which were lower. In the Blount blends without ryegrass at month 6, *H* was higher in all blends than in the control. In the blends with ryegrass at month 6, *H* was lower than the control in all blends, except the IGS-2, AGS-1, and AGS-2 blends made with Blount soil. Interestingly, the lowest *H* (i.e. 0.70) at month 6 occurred in Matawan soil blended with silica sand and seeded with ryegrass. Overall it should be noted that at week 4 and month 6, in both soils, *H* was generally lower in the ryegrass treatments than those without. It is likely that bacterial diversity is lower in the ryegrass treatments due to a depletion of available soil nutrients as a result of the plant growth.

Sequence analysis of isolates. To gain a better understanding of the dominant culturable members of the bacterial community in the SFS blends, 16S rDNA from the isolates was PCR-amplified and sequenced. The 16S rDNA sequences were then compared to bacterial reference strains to determine the phylogenetic relationships among the isolates. Phylogenetic trees for the Matawan and Blount soils and blends are shown in Figs. 1A and

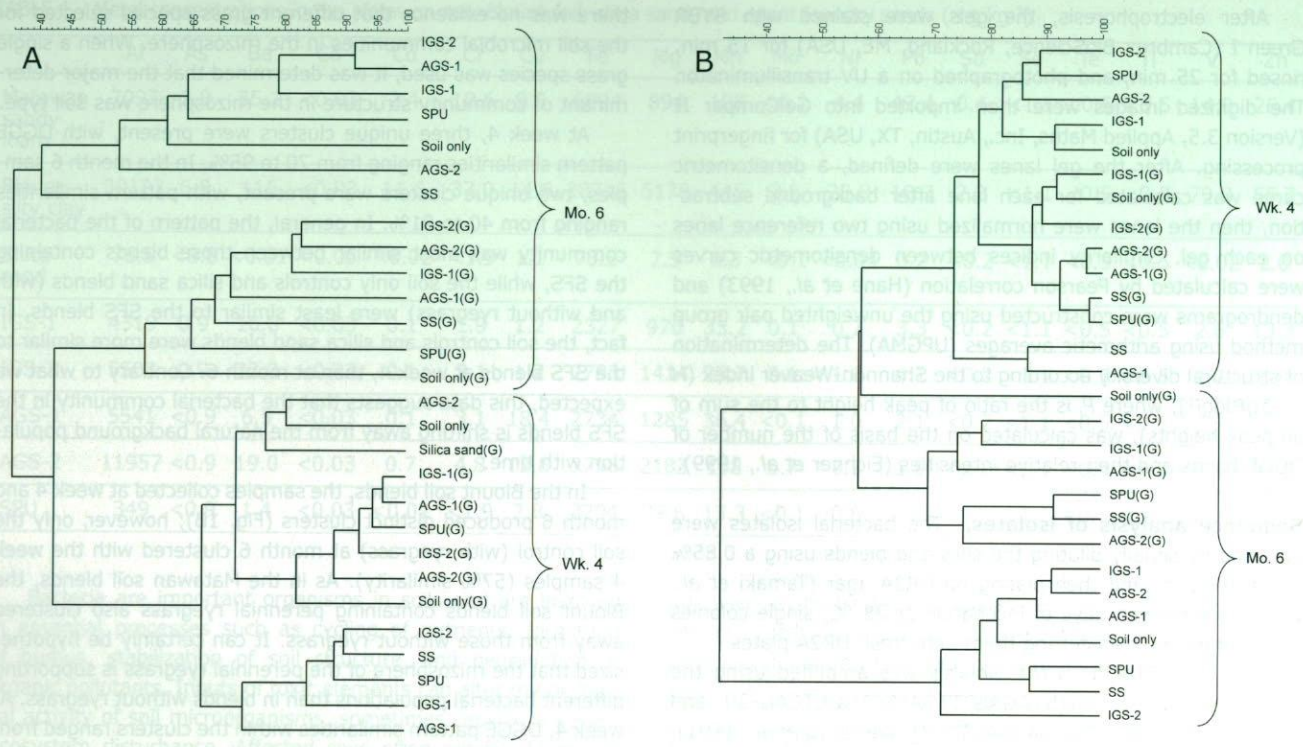

FIG. 1 - Cluster analysis of DGGE patterns of 16S rDNA fragments from the Matawan sandy loam (A) and the Blount silty clay (B) amended with spent foundry sands, with and without perennial ryegrass, at week 4 and month 6. SS: silica sand, AGS: aluminum green sand, IGS: iron green sand, SPU: steel phenolic urethane no-bake sand. The G in parentheses indicates those treatments that contained perennial ryegrass.

1B, respectively (month 6 data only). A total of 157 isolates from the Matawan and 152 isolates from the Blount soils and blends were examined. The isolates from the soils and blends were related to the following phylogenetic groups: Actinobacteria, Bacilli, Proteobacteria (α, β and γ subgroups) and Bacteroidetes. In the Matawan soil and blends (with and without ryegrass), 32, 23, 38, and 6% of the 16S rDNA sequences were related to Actinobacteria, Bacilli, Proteobacteria and Bacteroidetes, respectively. In the Blount soil and blends (with and without ryegrass), 61, 16, 19, and 4% of the sequences were related to the respective groups.

The isolates from the Matawan soil and blends were evenly distributed among those treatments with and without grass. The largest cluster was associated with Actinobacteria, with 36 of 50 isolates closely related to *Arthrobacter globiformis*, *Arthrobacter ramosus*, and *Arthrobacter histidinolovorans* (Fig. 2).

TABLE 2 - Effect of the treatments based on the Shannon-Weaver index of diversity, *H*.

		Week 4		Month 6	
Soil	Treatment†	No grass	Grass	No grass	Grass
Sandy loam	Soil only	1.29	1.23	1.45	1.14
	Silica sand	1.41	1.21	1.44	0.70
	IGS-1	1.43	1.20	1.30	1.08
	IGS-2	1.50	1.31	1.43	1.08
	AGS-1	1.39	1.27	1.37	1.02
	AGS-2	1.24	1.27	1.48	0.83
	SPU	1.18	1.26	1.46	1.06
Silty clay	Soil only	1.38	1.30	1.22	1.13
	Silica sand	1.39	1.20	1.47	1.12
	IGS-1	1.48	1.22	1.36	0.95
	IGS-2	1.55	1.29	1.42	1.35
	AGS-1	1.29	1.19	1.39	1.31
	AGS-2	1.37	1.18	1.36	1.36
	SPU	1.43	1.08	1.47	1.06

† IGS, Iron green sand; AGS, Aluminum green sand; SPU, Steel phenolic urethane no-bake sand

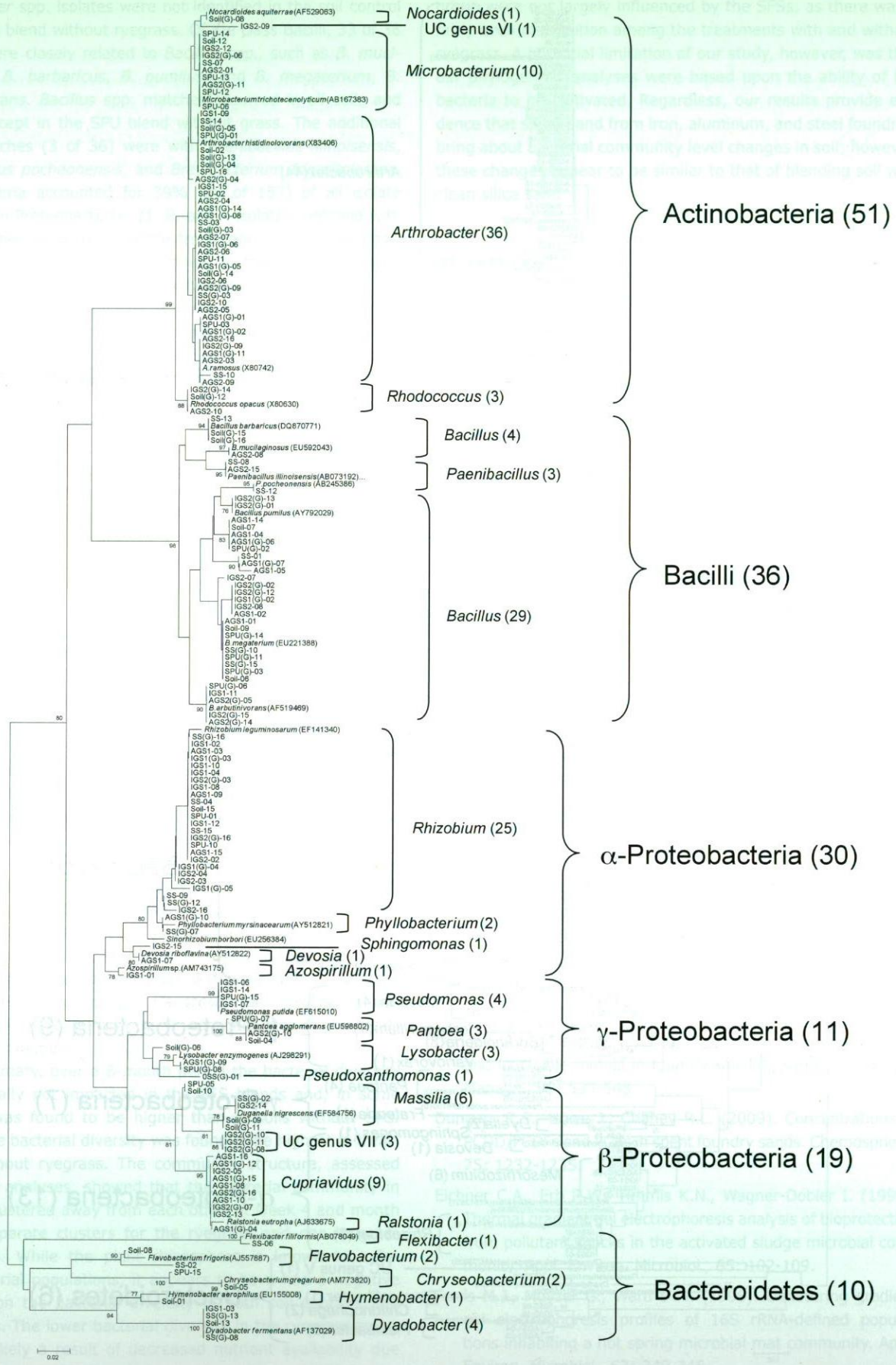

FIG. 2 - Phylogenetic tree of bacterial isolates from the Matawan sandy loam amended with spent foundry sands, with and without perennial ryegrass. Month 6 data is presented. SS: silica sand, AGS: aluminum green sand, IGS: iron green sand, SPU: steel phenolic urethane no-bake sand. The G in parentheses indicates those treatments that contained perennial ryegrass.

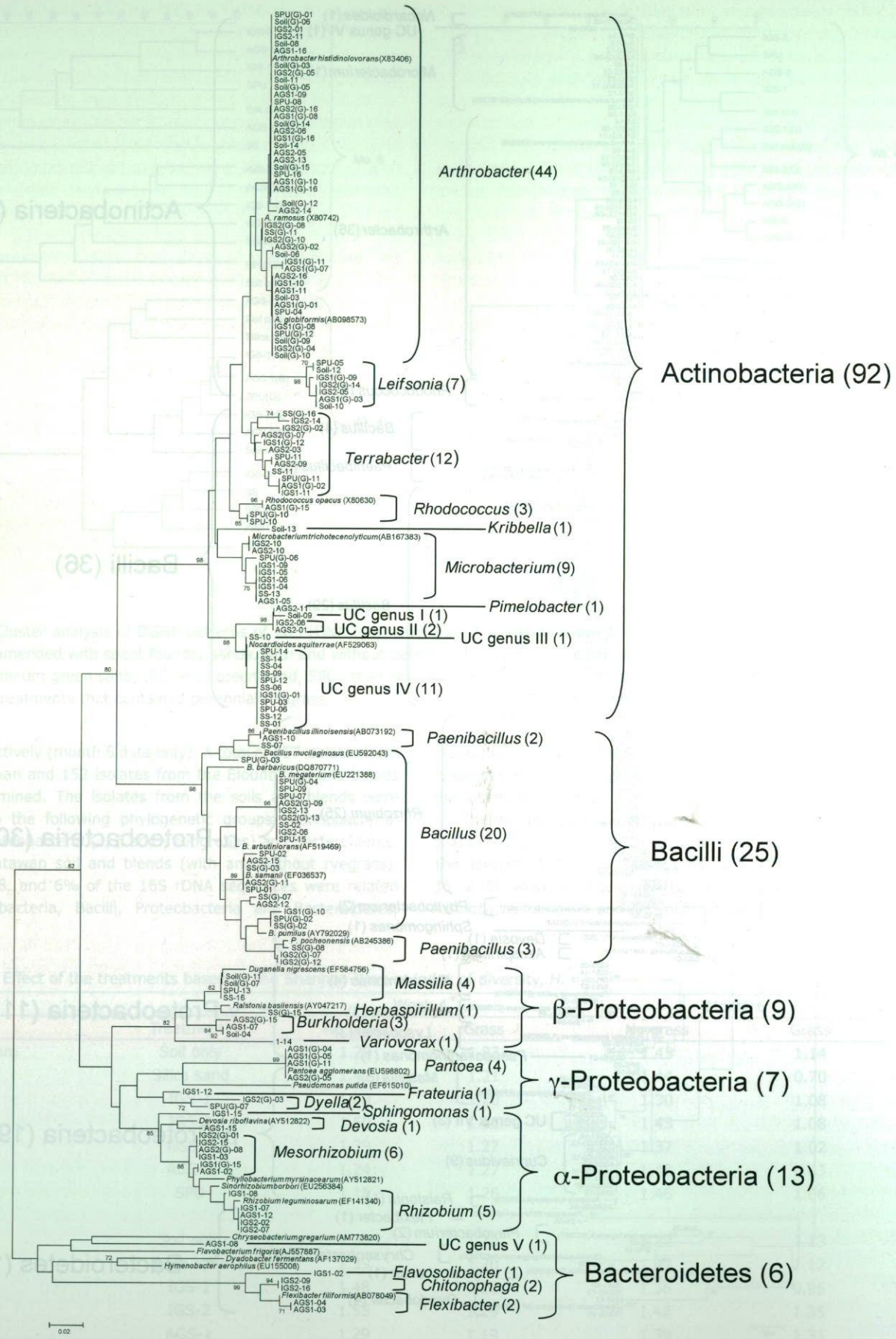

FIG. 3 - Phylogenetic tree of bacterial isolates from the Blount silty clay amended with spent foundry sands, with and without perennial ryegrass. Month 6 data is presented. SS: silica sand, AGS: aluminum green sand, IGS: iron green sand, SPU: steel phenolic urethane no-bake sand. The G in parentheses indicates those treatments that contained perennial ryegrass.

Arthrobacter spp. isolates were not identified in the soil control and AGS-1 blend without ryegrass. Of the class Bacilli, 33 of 36 isolates were closely related to *Bacillus* spp., such as *B. mucilaginosus*, *B. barbaricus*, *B. pumilus*, and *B. megaterium*, *B. arbutinivorans*. *Bacillus* spp. matches occurred in all soils and blends, except in the SPU blend without grass. The additional Bacilli matches (3 of 36) were with *Paenibacillus illinoisensis*, *Paenibacillus pocheonensis*, and *Brevibacterium frigoritolerans*. Proteobacteria accounted for 39% (60 of 157) of all isolate matches. In Proteobacteria, 21, 8, and 4 isolates matched with the α, β and γ subgroups in the treatments without ryegrass, while 9, 11, and 7 isolates matched in the treatments with ryegrass, respectively. In the α subgroup, the majority of the isolates matched with *Rhizobium leguminosarum*; however, only 7 isolates matched in the ryegrass treatments versus 18 in the treatments without ryegrass. The β and γ subgroup matches were predominantly affiliated with *Cupriavidus*, *Massilia*, and *Pseudomonas*, *Erwinia*, and *Lysobacter* spp., but were only found in some of the blends. The majority of the Bacteroidetes matches (i.e. *Chryseobacterium*, *Dyadobacter*, *Flavobacterium*, *Flexibacter*, and *Hymenobacter* spp.) were found in the soil control and silica sand blend without ryegrass.

In the Blount soil treatments (Fig. 3), Actinobacteria was the largest cluster in the phylogenetic tree, accounting for 62% (92 of 152) of all isolate matches. Almost one half of the isolates within this phylum were affiliated with the genus *Arthrobacter*, which were found in all of the treatments (with and without ryegrass), except the silica sand blend without ryegrass. Additionally, *Kribbella*, *Leifsonia*, *Microbacterium*, *Pimelobacter*, *Rhodococcus*, and *Terrabacter* spp. and four unclassified genera were identified. In the treatments with ryegrass, none of the isolates matched with the genera *Kribbella* and *Pimelobacter*. In the class Bacilli, 20 of 25 isolates matched closely with *Bacillus* (i.e. *B. mucilaginosus*, *B. megaterium*, *B. samanii*, and *B. pumilus*), with the rest of the isolates matching with *Paenibacillus* (i.e. *P. illinoisensis* and *P. pocheonensis*). These isolates were not identified in the soil only controls, but were found in most of the SFS blends. Compared to the Matawan treatments, fewer of the isolates were identified as Proteobacteria; in total, only 13, 9, and 7 isolates matched with the α, β and γ subgroups, respectively. The most abundant Proteobacteria isolates were from the following genera: *Mesorhizobium*, *Rhizobium*, *Massilia*, and *Pantoea*. In the Blount treatments without ryegrass, 6 isolates were identified as Bacteroidetes (*Chitonophaga*, *Flavosolibacter*, *Flexibacter*, and one unclassified bacterium); none were identified in the treatments with ryegrass.

In summary, over a 6-month period the bacterial diversity was generally not impacted in the SFS blends and, in some cases, it was found to be higher than in soils without SFS. Overall, the bacterial diversity was found to be highest in treatments without ryegrass. The community structure, assessed via cluster analyses, showed that the bacterial community in the soils clustered away from each other at week 4 and month 6, with separate clusters for the ryegrass and non-ryegrass treatments. While the plant rhizosphere is known to influence bacterial populations, it appears to have had a negative impact upon the bacterial diversity in both soil controls and SFS blends. The lower bacterial diversity in the ryegrass treatments is likely a result of decreased nutrient availability due to the plant growth. The phylogenetic analysis of 309 bacterial isolates revealed that the soil controls and SFS blends were dominated by Actinobacteria, Proteobacteria (α, β and γ subgroups), and Bacilli. The genera within each phylogenetic group were not largely influenced by the SFSs, as there was a fairly even distribution among the treatments with and without ryegrass. A potential limitation of our study, however, was that our phylogenetic analyses were based upon the ability of the bacteria to be cultivated. Regardless, our results provide evidence that spent sand from iron, aluminum, and steel foundries bring about bacterial community level changes in soil; however, these changes appear to be similar to that of blending soil with clean silica sand.

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