Title: **Selective progressive response of soil microbial community to wild oat roots**

Running head: Dynamic rhizosphere microbial community composition

Authors: Kristen M. DeAngelis[1,2], Eoin L. Brodie[1], Todd Z. DeSantis[1], Gary L. Andersen[1], Steven E. Lindow[2], Mary K. Firestone[1, 3*]

[1]Ecology Department, Lawrence Berkeley National Lab, Berkeley CA 94720; [2]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720; [3]Department of Environmental Science, Policy and Management, University of California, Berkeley; CA 94720;

*Corresponding author: 137 Mulford Hall, Department of Ecosystem Science, Policy and Management, University of California, Berkeley; CA 94720, Tel: (510) 643-2402, Fax: (510) 642-6847, email, mkfstone@nature.berkeley.edu

Running title: Rhizosphere soil microbial community dynamics

Journal: ISME Journal

Subject category: Microbial population and community ecology

Keywords: Rhizosphere soil/16S rRNA/microarray/PhyloChip/TRFLP/bacterial and archaeal populations

Abstract

Roots moving through soil enact physical and chemical changes that differentiate rhizosphere from bulk soil, and the effects of these changes on soil microorganisms have long been a topic of interest. Use of a high-density 16S rRNA microarray (PhyloChip) for bacterial and archaeal community analysis has allowed definition of the populations that respond to the root within the complex grassland soil community; this research accompanies previously reported compositional changes, including increases in chitinase and protease specific activity, cell numbers and quorum sensing signal. PhyloChip results showed a significant change in 7% of the total rhizosphere microbial community (147 of 1917 taxa); the 7% response value was confirmed by16S rRNA T-RFLP analysis. This PhyloChip-defined dynamic subset was comprised of taxa in 17 of the 44 phyla detected in all soil samples. Expected rhizosphere-competent phyla, such as Proteobacteria and Firmicutes, were well represented, as were less-well-documented rhizosphere colonizers including Actinobacteria, Verrucomicrobia and Nitrospira. Richness of Bacteroidetes and Actinobacteria decreased in soil near the root tip compared to bulk soil, but then increased in older root zones. Quantitative PCR revealed β-Proteobacteria and Actinobacteria present at about 10^8 copies of 16S rRNA genes g^{-1} soil, with Nitrospira having about 10^5 copies g^{-1} soil. This report demonstrates that changes in a relatively small subset of the soil microbial community are sufficient to produce substantial changes in function in progressively more mature rhizosphere zones.

Introduction

As roots move through soil they impact its physical, chemical and biotic characteristics, and these changes are accompanied by alterations in microbial community activity (Bringhurst *et al.*, 2001; DeAngelis *et al.*, 2008). Soil that is directly influenced by roots, the rhizosphere, can make up a substantial volume of temperate zone soils, with root influence extending to meters depth (Lynch and Whipps, 1990). Root movement through soil creates dynamic environmental gradients that are constantly reiterated with new root growth. A root moving through "bulk soil" introduces labile carbon and nutrients, creates water conduits, and deposits antimicrobial compounds and hormones (Brimecombe *et al.*, 2001; Bringhurst *et al.*, 2001; Hawes *et al.*, 1998; Hawkes *et al.*, 2007) across temporal scales of hours to days (Jaeger *et al.*, 1999; Lubeck *et al.*, 2000). As many soil microbes are carbon limited (Paul and Clark, 1996), they may be expected to respond quickly to root-induced changes in soil chemistry and nutrient status by reproducing and increasing in activity (Heijnen *et al.*, 1995; Herman *et al.*, 2006; Jaeger *et al.*, 1999). This enhanced microbial activity may accelerate nutrient cycling as well as other functions such as pathogenesis.

Studies of microbial community profiles posit that there are substantial impacts of roots on bacterial and archaeal communities in soil (Lynch and Whipps, 1990), though there is little direct evidence for changes occurring in specific indigenous soil populations. Previous studies on rhizosphere microbial community dynamics have used fingerprinting methods such as DGGE and T-RFLP along with diversity indices such as Shannon-Simpson's to describe these communities (Pett-Ridge and Firestone, 2005; Yang and Crowley, 2000). Some taxonomic resolution has been achieved by primer-specific DGGE targeting α-Proteobacteria, β-

Proteobacteria and Actinomycetes, where the relative abundance of several Proteobacteria and some Actinobacteria were observed to increase in the rhizosphere (Costa *et al.*, 2006). While such studies suggest that selective growth of specific groups of bacteria occurs in the rhizosphere, the sheer diversity of soil microbiota and the limited resolution of available methods have limited such assessments.

Soil genomic DNA re-association kinetics predict that about 10^6 different genomes per gram soil comprise temperate, uncontaminated soil microbial communities (Curtis and Sloan, 2005; Torsvik *et al.*, 2002), but most methods are only capable of resolving orders of magnitude fewer taxa. Terminal restriction fragment length polymorphism (TRFLP) of 16S rRNA can resolve about 10^2 operational taxonomic units (OTUs); OTUs are usually assumed to be distinct phylotypes, or taxa, for methodological purposes. While this is a robust and reproducible method, it yields relatively low-resolution information about microbial community composition (Osborn *et al.*, 2000). Clone library analysis has expanded our knowledge of rhizosphere community composition and allowed documentation of uncultivable taxa but is limited to describing only those taxa most common in the sample. In a recent meta-analysis of 19 clone libraries from 14 plant species, over 1200 distinguishable taxa from 35 different taxonomic orders were revealed (Hawkes *et al.*, 2007). Proteobacteria dominated the rhizosphere in 16 of 19 studies included, which is in keeping with other suppositions of proteobacterial dominance of rhizosphere communities, presumably due to their relatively rapid growth rates (Atlas and Bartha, 1993). However, this analysis also uncovered a large number of other phyla, suggesting that root stimulation of microbial communities may be more complex than that predicted by the model of “hungry heterotrophs” simply responding to rhizosphere carbon deposition.

This research employs a method capable of documenting community composition with resolution that more closely approaches the expected diversity in soil. High-density 16S rRNA PhyloChip has the resolution of almost 10^4 taxa, and the ability to identify individual taxa varying by over five orders of magnitude in abundance (Brodie *et al.*, 2006). This PhyloChip community analysis examines how microbial community composition changes in response to the environmental changes accompanying root movement through soil. The exploration of soil community dynamics in response to roots complements our previous reports indicating that roots moving through soil result in substantial changes in microbial function (DeAngelis *et al.*, 2008; Herman *et al.*, 2006). These data may provide the most comprehensive analysis to date of the succession of a rhizosphere microbial community, additionally articulating the magnitude and identity of changing component populations within the rhizosphere communities.

Methods

Preparation of soils, plants and microcosms

Soils were collected from the growing zones of the annual graminoid *Avena fatua* at the University of California Hopland Research and Extension Center (Hopland, Ca.). This soil is a medium-texture loam derived from hard sandstone and shale, classified as an ultic haploxeralf (Waldrop *et al.*, 2000). Soils were collected to a depth of 10 cm, and immediately transported to the lab, where they were sieved to 2 mm, homogenized and hydrated to 50% water holding capacity just prior to packing into microcosms.

Microcosms (Figure 1) were employed as previously described (DeAngelis *et al.*, 2008; Jaeger *et al.*, 1999). Briefly, seeds of *Avena fatua* (Valley Seed Service, Fresno CA) were pre-

germinated prior to planting under a slow drip of tap water for 4 days in darkness. Plants were watered until soil reached water holding capacity with tap water every second or third day, depending upon the weather to avoid saturation, and incubated in the greenhouse under ambient light conditions. After 8 weeks of growth, the experimental side chambers were filled with soil and the microcosms were inclined at a 45-degree angle so the roots would grow along one face of the microcosm. After 8 days of growth the front plate was carefully removed to expose the roots, and samples of 4 types of soil were harvested. Bulk soil was excised at least 4 mm away from any roots, and soils within 2mm of the root surface were considered rhizosphere soil and extracted with a scalpel from three rhizosphere zones: root tip rhizosphere soil was located 0-4cm from the root tip, root hair rhizosphere soil was 4-8cm from the root tip, and mature root rhizosphere soil was 8-16cm from the root tip.

Determination of live cell abundance by direct count

The number of live bacteria were determined using the BacLight Bacterial Viability Kit (Molecular Probes Inc., Eugene OR) (Boulos *et al.*, 1999). Serial dilutions of fresh soil were immediately made in phosphate buffered saline, sonicated, stained, and counted within 48 hours of harvest. Cells were stained with SYTO9 and propidium iodide nucleic acid stains; SYTO9 stains only cells with intact membranes in the presence of propidium iodide, indicating living cells. Live cells were counted using epiflourescence microscopy on a Leica DMRX with a 630X fluorescence oil-immersion lens (Leica Microsystems, Bannockburn, IL). At least two slides were prepared per sample, for different dilutions, and ten fields of view were counted per slide. Numbers of live bacteria are presented as cells g^{-1} dry soil.

PCR amplification of 16S ribosomal RNA genes

DNA was pooled from three separate extractions from about 250 mg (fresh weight) of soil using a modified bead beating method (Brodie *et al.*, 2002; Griffiths *et al.*, 2000). Soils were added to CTAB (hexadecyltrimethylammonium bromide) extraction buffer, consisting of equal volumes of 10% CTAB in 0.7M NaCl and 240mM potassium phosphate buffer pH 8.0, and then bead-beaten by adding 0.1 mm glass and 0.5mm zirconia/silica beads (Biospec Products Inc, Bartlesville OK), 0.5ml phenol:chloroform:isoamylalcohol (25:24:1) and shaken in a FastPrep Instrument (Qbiogene, Inc., Irvine CA) at 5.5m s^{-1} for 30s. Following bead-beating, soils were extracted with an equal volume of chloroform:isoamylalcohol (24:1), DNA was precipitated with PEG6000/NaCl solution and following ethanol washing, was resuspended in water. One-tenth dilutions of soil DNA extracts were used as a template for amplification of bacteria and archaea 16S rRNA genes for TRFLP and PhyloChip analysis. The primers 8F and 1492R were used for TRFLP, while the primers 8F* and 1492R for bacteria and 4Fa and 1392R were used for archaea for PhyloChip analysis (Table 1). PCR reactions were performed in 50µl using Takara ExTaq with 3µM of each primer, 50µg BSA, and 2u of DNA polymerase (Takara Mirus Bio Inc., Madison WI). Eight replicate PCR amplifications were performed at a range of annealing temperatures from 52-62°C in a BioRad iCycler (BioRad, Hercules CA) with an initial denaturation (5min) followed by 30 cycles for TRFLP and 25 cycles for PhyloChip of 95°C (30sec), annealing (30sec), and 72°C (90sec), and a final extension of 72°C (8min). PCR reactions were run on 1% TAE agarose gel to check for products. Bacterial 16S rRNA PCR product was cleaned up using Qiagen PCR Miniprep Kit (Qiagen Sciences, Valencia, CA), while archaeal 16S rRNA PCR product was gel purified and cleaned up using the MoBio Gel Purification Kit (MoBio Laboratories, Inc., Solana Beach CA).

Microbial community analysis by 16S rRNA PhyloChip

For application onto the PhyloChip, PCR products were concentrated to 1000 ng (bacterial) or 200 ng (archaeal), then fragmented, biotin labeled and hybridized to PhyloChips as previously described (Brodie *et al.*, 2006). The microbial community analysis was resolved as a subset of 8743 taxa on the PhyloChip, where each taxon is represented by a set of an average of 24 perfect match-mismatch probe pairs (minimum 11, maximum 30). For a taxon to be reported in this analysis, 90% of probe pairs in its set (probe fraction, or pf) must have (1) a perfect match intensity at least 1.3 times the mismatch, and (2) difference between perfect match and mismatch intensity that are 130 times the square of background intensity. Hybridization scores for a taxon are reported for all samples if at least one sample out of the twelve has pf>0.9; for definition of the dynamic subset, this was refined to three replicated samples out of twelve, which is analogous to TRF calling (Zak *et al.*, 2006). Hybridization scores are an average of the difference between perfect match and mismatch fluorescent intensity of all probe pairs excluding the highest and lowest; they were normalized to an average of 2500 au based on internal standards and are reported as arbitrary units (au). In presenting relative abundances of reported taxa, hybridization scores were converted to 16S copy number based on the empirically determined log-linear relationship between copy number of applied 16S rRNA PCR product and hybridization score (Brodie *et al.*, 2007).

Microbial community analysis by 16S rRNA TRFLP

Bacterial 16S rRNA gene products were amplified by PCR as above with the forward primer modified to contain a 6-carboxyfluorescein (6-FAM, Sigma-Genosys, The Woodlands

TX) for detection in capillary electrophoresis. About 0.5µg of PCR product was digested overnight with the restriction enzyme MspI in a reaction mixture containing 2u enzyme and appropriate buffers. Digested DNA was precipitated, resuspended in formamide and mixed with GeneScan 500-ROX size standards (Applied Biosystems, Foster City CA)(Brodie *et al.*, 2002). Immediately before electrophoresis, amplicons were denatured and electrophoresis was performed on an ABI 3100 automated capillary sequencer (Applied Biosystems, Foster City CA). In culling terminal restriction fragments (TRFs) to use in the final data analysis, peaks were eliminated that were present in only one replicate, that had peak heights below 50 fluorescent units, or sizes outside of the range of the ROX size standard (Blackwood *et al.*, 2003). TRF peak heights were normalized, and TRF sizes were expressed in base pairs to the nearest base.

Quantitative PCR of dynamic taxa and groups

Primers were chosen or designed to target specific groups and taxa identified on the PhyloChip (Table 1). Designed quantitative real-time PCR (qPCR) probes were based on PhyloChip probes using the Greengenes web application (DeSantis *et al.*, 2006), checked for utility using Primer3 (Rozen and Saletsky, 2000), checked for specificity using RDP Probe Match (Cole *et al.*, 2005) and manufactured by IDT DNA (Integrated DNA Technologies DNA, Skokie IL). All qPCR was performed on an iCycler iQ real-time detection system (Applied Biosystems, Foster City CA) and used the Qiagen SYBR green kit (Qiagen Sciences, Valencia CA). 20ul reactions contained SYBR green 2x master mix, 6pmol each of forward and reverse primer, 10nM fluorescein, and one-tenth dilutions of soil DNA extract in nuclease free water. Conditions began with hot-start activation at 95°C (7 min), followed by 40 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (75 sec), and a data acquisition step at 78°C (10 sec) empirically

determined and optimized using melt curve analysis. Standard curves were run in parallel corresponding to a range of 10^8 to 10^1 copies ul^{-1}. Standard curve regression coefficients were consistently above 0.99, and melt curve analysis verified a single amplicon per reaction.

Statistical analysis

Descriptive statistics were performed using JMPIN (SAS Institute, Inc., Cary NC), and multivariate statistics were performed using PCOrd (MjM Software, Gleneden Beach, OR). The array intensities for all taxa were normally distributed based on a KSL Goodness-of-Fit test ($p<0.01$). Application of an adjusted p-value is too strong a restriction on the community data for these purposes (Yang and Speed, 2003), and because a stricter definition of the dynamic community does not substantially change the results or the variance explained, statistics were performed using a p-value of 0.05. To examine only the taxa that were major contributors to the change in community, we used two different methods due to the differences in size of the data sets. For TRFLP, 132 ANOVAs were performed in JMPIN; this method was not possible for the larger PhyloChip data set for which 3 two-tailed paired t-tests were executed between the bulk soil and each rhizosphere root zone (root tip, root hairs, or mature root). We defined the "dynamic subset" of the community as comprised of taxa having at least one significant paired t-test when three paired t-tests were performed. For ordination of whole communities, principle components analysis was chosen because it is best suited for data with an approximate linear relationship (McCune and Grace, 2002); in this case, the gradient is that of root exudate deposition with root age (Jaeger *et al.*, 1999). To estimate richness (S), we used a probe fraction value of 0.9 as a cutoff, below which the taxa was deemed absent. Previously probe fraction was found to correlate well with richness patterns displayed by clone library analysis (DeSantis *et al.*,

2007). For each root zone, we calculated differences in hybridization scores for each rhizosphere root zone from bulk soil, and then converted the difference to rRNA gene copies. Numbers of individuals (N) are based on live cell counts.

Results

Cell density in the rhizosphere compared to bulk soil

Significantly more live cells were detected in the rhizosphere compared to bulk soil (Figure 2). As many as tenfold more cells were detected in the root hairs and the root tip rhizosphere compared to bulk soil, with mature root rhizosphere soil having significantly higher cell density than bulk but less than root hairs or root tip ($p<0.0001$). The rate of *Avena fatua* root growth in experimental microcosms was approximately 2 cm day^{-1} (Herman *et al.*, 2006), and roots up to 16cm were surveyed, assessing changes in the rhizosphere over about 8 days.

16S rRNA PhyloChip community analysis

Of the possible 8743 resolvable taxa on the PhyloChip, we detected 2595 that had a positive probe fraction ($pf>0.9$) in at least one of the twelve samples. For the whole community analysis, we examined only taxa that were present (had a positive probe fraction) in all three replicates in any one root zone, defined as replicated taxa; 1917 taxa fit this criteria (Table S1). That more than 600 taxa were eliminated due to non-replication suggests high biological variability, and more replicates would likely put the total richness of these soils well above the 1917 taxa observed.

For each of the observed 1917 taxa, we examined the change in relative abundance compared to the background bulk soil. The fold difference in 16S rRNA gene copy number of each taxon in the root tip, root hairs, or mature root zone rhizosphere relative to the bulk soil background is displayed in a histogram in which the taxa are ranked by decreasing relative abundance (Figure 3A). Most populations responded positively to the presence of the root in all root zones compared to bulk soil. A small portion of the taxa exhibited more than a 2-fold increase compared to bulk soil, while a few taxa exhibited large (up to 10-fold) increases relative to bulk soil. The dynamic subset of the community contains all taxa that showed significant response to the presence of the root ($p<0.05$), and are summarized in Table 2. The dynamic subset is comprised of 147 significant taxa and represents 7% of the total community, falling into 17 phyla of the 44 initially detected phyla. Seven of these phyla contain taxa that significantly decreased in the root tip rhizosphere zone compared to bulk soil (Figure 3B). Taxa in the dynamic subset all eventually become successful root colonizers, since in general, the longer the community was exposed to the root, the greater the increase in relative abundance of the taxa. Representative response patterns of individual taxa can be discerned by examining the ten individual taxa that changed the most within the dynamic subset (Figure 3C). The different response patterns of these taxa show discontinuous positive responses to the root tip as well as negative responses to the newly arrived root. Only three taxa, TM7 AB100499, Acidobacteria AF498753, and Bacteroidetes AB023506 show a progressively positive response to the aging root zones.

Principal components analysis performed for the dynamic subgroup of 147 taxa explained 78% of the dataset variance in two axes (Figure 4A). Differences in the root zone communities could be discerned, indicating that the dynamic subset is comprised of taxa that

responded strongly to the presence of the root. Multi-response permutation procedure determined that there is a greater than 90% chance that there are differences between the microbial communities in the four soil zones ($p=0.099$).

Twenty of a possible 309 archaeal taxa were detected by PhyloChip analysis of these soils, and of these, 19 were not present with $pf>0.90$ in all replicates, thus excluding them from the replicated data set of 1917 taxa (Table S1). However, the archaea examined at all probe fractions revealed a strong positive response to the presence of the root (Figure 4B).

To understand whether the changing relative abundances in taxa between different root zones were manifested in changes in richness, we enforced a cutoff probe fraction of 0.9, below which we assumed that the taxon was not present. This revealed differences in richness between root zones, as there were more taxa in the root hairs and mature root compared to bulk soil. The root tip actually shows a slight decrease in richness compared to bulk soil (Figure 5), these differences were not significant overall, but were manifested in a decrease in richness for the Bacteroidetes overall ($p=0.083$) and the Actinobacteria in the dynamic subset community ($p=0.049$). Additionally, it was the sum of many diverse phyla ("all others", Figure 5) more than the major phyla that appeared to contribute most to the increased whole-community richness in the root hairs ($p=0.065$) (Figure 5, Table S1).

TRFLP community analysis of rhizosphere diversity

A total of 132 TRFs were resolved among all samples by 16S rRNA TRFLP. Of these TRFs, nine had a significantly different relative abundance as determined by ANOVA ($p<0.05$) in the presence of the root (data not shown). It is likely each TRF represents more than one bacterial species or taxa (Costa *et al.*, 2006; Yang and Crowley, 2000), but we can assume that

all TRFs represent on-average the same number of taxa. About 7% of species present in this analysis (9 of 132 TRFs) had an abundance that was significantly affected by the root.

Quantitative PCR of dynamic taxa groups

Quantitative PCR was performed to assess the actual abundance of certain taxa that were observed by PhyloChip analysis to be most responsive to the root (Table S1, Table 2). We used specific primer sets to examine the β-Proteobacteria and Actinobacteria, which were commonly represented, and Nitrospira, having only one represented taxon (Table S1). While the variance in estimate of taxa abundance was too large to resolve differences of any of the groups between the different root zones, the absolute abundance of β-Proteobacteria and Actinobacteria were both about 10^8 16S copies g^{-1} soil, while Nitrospira were about 10^5 copies g^{-1} soil.

Discussion

PhyloChip analysis reveals that a diverse array (17 phyla) of bacterial and archaeal populations changed in relative abundance in the rhizosphere, which is somewhat at odds with the long-held assumption that the fast-growing, easily cultivable Proteobacteria are the dominant rhizosphere colonizers (Hawkes *et al.*, 2007; Lynch and Whipps, 1990; Paul and Clark, 1996). We have previously detected increased acyl-homoserine lactone (AHL) abundance in the rhizosphere compared to bulk soil (DeAngelis *et al.*, 2007; DeAngelis *et al.*, 2008); AHL-mediated quorum sensing occurs exclusively within the phylum Proteobacteria (Loh *et al.*, 2002). While this suggests that proteobacteria increase in numbers in the rhizosphere compared to bulk soil, the proteobacteria as a group were only one phylum among many represented in the

dynamic subset of 147 taxa. Our data suggest that there is a diverse dynamic subset of the soil bacterial and archaeal community that is specifically stimulated by the root; the richness of the responsive subset spans about 7% of the total community and encompasses up to 17 different phyla.

Based on richness estimates from the PhyloChip, the Firmicutes, α-Proteobacteria and Actinobacteria comprised the greatest portion of taxa that changed significantly in relative abundance in response to the root (Table 2). A greater portion of the Actinomycetes (16.9%) and α-Proteobacteria (11.4%) changed in response to the root than the overall percentage of taxa in the dynamic subset (7.7%); the Firmicutes were about as responsive to the root as the dynamic taxa as a whole (6.9% compared to 7.7% overall) (Table 3). Well known decomposers (Paul and Clark, 1996), the Actinobacteria are considered prototypical microbial k-strategists (Atlas and Bartha, 1993); as such it is somewhat surprising that so many members of this group would increase in rhizosphere dominance. However, a strong actinobacterial response to the *Avena* root may explain some of the increase in exoenzyme activity that we have recently reported (DeAngelis *et al.*, 2008). Our results suggest that the Actinomycetes and α-Proteobacteria include taxa that are exceptionally rhizosphere-competent.

Many microorganisms remain known only by 16S rRNA since they resist culturing efforts (Macrae *et al.*, 2000), and community analysis by PhyloChip yields insight into the lifestyle of such organisms. Verrucomicrobia generally resists culturing (Sangwan *et al.*, 2005), but culture-independent analyses reveals that this group is present in many soils. This group has been occasionally observed in rhizosphere soils from aerated systems (Ulrich and Becker, 2006), and seems to have representatives that respond positively to the rhizosphere in this study (Table 2). A recent study, using ^{13}C-CO_2 to pulse-label plant root exudates and thus identify rhizosphere

primary producers, found mostly Proteobacteria with many fewer Actinobacteria and Acidobacteria (Vandenkoornhuyse *et al.*, 2007). Though the proteobacteria as a group are often considered prototypical fast-growers, only the β- and γ-Proteobacteria are well represented by culturing efforts and known fast-growers (Paul and Clark, 1996; Schmidt *et al.*, 2007). The functionally diverse Acidobacteria phylum also seems to have a few members that are strongly rhizosphere competent (Vandenkoornhuyse *et al.*, 2007), but whose role in rhizosphere processes remains to be demonstrated. A related study of cultivated strains from *Avena* rhizosphere soil uncovered many diverse and previously uncultured α-Proteobacteria (DeAngelis *et al.*, 2008), further suggesting that the α-Proteobacteria may contain many rhizosphere-competent taxa. Vandenkoornhuyse and colleagues also found five potentially new phylotypes by stable isotope probing (Vandenkoornhuyse *et al.*, 2007). Taken together, these observations suggest that roots stimulate a broad diversity of the soil microbial community, influencing taxa that are as yet unknown and undefined.

While the older root zones exhibited a fairly typical rhizosphere effect, having increasing taxa abundance, the root tip was somewhat of an anomaly. The number of cells in soil near the root tip was significantly higher than in bulk soil (Figure 2), however the number of taxa present was not higher (Figure 5). Production of plant defense compounds by border cells in the root tip may be responsible for the somewhat decreased richness in this zone (Hawes *et al.*, 1998). Phyla that include typical plant pathogens and symbionts, such as the Pseudomonadales, Rhizobiales, and Bradyrhizobiales, were among taxa that decreased in relative abundance in the root tip (Table 2), as well as many taxa in the Actinobacteria, Bacteroidetes, and α-Proteobacteria. However, as the root tip moved through soil, these populations all eventually recovered, since almost the entire community responded positively to the presence of the mature root (Figure 3);

the exceptions were all four Bacilli (phylum Firmicutes) whose population sizes remained low. The effect of root defense compounds produced in the root tip on selective microbial populations merits further investigation.

A few taxa that showed a positive response to the root have not been previously f ound to be competent for rhizosphere growth because they are present only in low relative abundance; the ability to detect such taxa is one of the major advantages of community analysis by PhyloChip. Nitrospira accounted for about 10^5 cells g^{-1} soil compared to the 10^{9-10} total bacteria, and one taxon of Nitrospira was identified as responding strongly positively to the presence of the root in this study. To our knowledge, all studies on Nitrospira are from water-saturated (wetland or rice) rhizosphere soil (Briones *et al.*, 2003; Ikenaga *et al.*, 2003; Kowalchuk *et al.*, 1998). Our results also suggest a Crenarchaeal population in soil that responds strongly to the root (Table 2, Figure 4). The Crenarchaeota include ammonia oxidizers that may be functional in mesophilic, aerobic soil environments (Treusch *et al.*, 2005). More focused investigation is required before concluding that Nitrospira or Archaea contain taxa that are strong rhizosphere responders. PhyloChip detection of Nitrospira, verified by qPCR to be present at about 10^5 per gram soil, confirms the capacity of this method to reliably detect taxa in low relative abundance, down to a detection limit of about 0.01% of the total community (Brodie *et al.*, 2007).

Changes in 16S rRNA gene copy numbers suggest that a large fraction of the rhizosphere community is two to ten times the relative abundance of the bulk soil (Figure 3), and community analysis reveals that the root affects a dynamic subset community in a coordinated way (Figure 4). The dynamic subset is not only increasing in relative abundance, but taxa in a diverse, yet defined, subset are differentially responding to the characteristics of specific root zones as the root moves through soil. Compositional differences in the rhizosphere community compared to

bulk soil and between different root zones point to a fraction of the soil microbial community that is especially rhizosphere competent (Folman *et al.*, 2001; DeAngelis *et al.*, 2006; Nunan *et al.*, 2005). These compositional changes accompany large changes in soil function associated with rhizosphere N cycling (DeAngelis *et al.*, 2006; DeAngelis *et al.*, 2008). The linkage suggested between the changes in community composition reported here and the changes in N-cycling previously reported clearly deserve further exploration.

Acknowledgements

The authors would like to gratefully acknowledge Ellen Simms for thoughtful discussions regarding statistical analysis of the data, and Yvette Piceno for technical assistance with the PhyloChip. This research was funded in part by the Environmental Protection Agency Science To Achieve Results Program (EPA-STAR) Grant and the National Science Foundation Doctoral Dissertation Improvement Grant to KMD. This work was also supported by California Experimental Station Project 6117-H to MKF. Additional work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Berkeley National Laboratory, under Contract DE-AC02-05CH11231 and was supported, in part, by the Program for Ecosystem Research (MFK, ELB, TZD, GLA).

References

Amann RI, Ludwig W, Schleifer KH (1995). Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. *Microbiol Reviews* **59**: 143-169.

Atlas RM, Bartha R (1993). *Microbial ecology: fundamentals and applications*. Benjamin/Cummings Publishing: Fonte, Ca.

Blackwood CB, Marsh T, Kim SH, Paul EA (2003). Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Appl Environ Microbiol* **69:** 926-932.

Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R (1999). LIVE/DEAD (R) BacLight (TM): application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* **37:** 77-86.

Brimecombe MJ, DeLeij FA, Lynch JM (2001). The effect of root exudates on rhizosphere microbial populations. In: Pinton R, Varanini Z and Nannipieri P (eds). *The rhizosphere: biochemistry and organic substances at the soil-plant interface.* Marcel-Dekker, Inc.: New York. pp 95-140.

Bringhurst RM, Cardon ZG, Gage DJ (2001). Galactosides in the rhizosphere: Utilization by *Sinorhizobium meliloti* and development of a biosensor. *Proc Natl Acad Sci USA* **98:** 4540-4545.

Briones AM, Okabe S, Umemiya Y, Ramsing NB, Reichardt W, Okuyama H (2003). Ammonia-oxidizing bacteria on root biofilms and their possible contribution to N use efficiency of different rice cultivars. *Plant and Soil* **250:** 335-348.

Brodie EL, DeSantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL, Hazen TC, Richardson PM, Herman DJ, Tokunaga TK, Wan JM, Firestone MK (2006). Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. *Appl Environ Microbiol* **72:** 6288-6298.

Brodie EL, DeSantis TZ, Parker JP, Zubietta IX, Piceno YM, Andersen GL (2007). Urban aerosols harbor diverse and dynamic bacterial populations. *Proc Natl Acad Sci USA* **104:** 299-304.

Brodie EL, Edwards S, Clipson N (2002). Bacterial community dynamics across a floristic gradient in a temperate upland grassland ecosystem. *Microb Ecol* **44**:260-270.

Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM *et al* (2005). The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucl Acids Res* **33:** D294-D296.

Costa R, Gotz M, Mrotzek N, Lottmann J, Berg G, Smalla K (2006). Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *Fems Microbiology Ecology* **56:** 236-249.

Curtis TP, Sloan WT (2005). Exploring microbial diversity - A vast below. *Science* **309:** 1331-1333.

DeAngelis KM, Ji PS, Firestone MK, Lindow SE (2005). Two novel bacterial biosensors for detection of nitrate availability in the rhizosphere. *Appl Environ Microbiol* **71:** 8537-8547.

DeAngelis KM. (2006). Microbial community ecology and bacterial quorum sensing as control points in rhizosphere nitrogen cycling. *Vol PhD.* University of California, Berkeley.

DeAngelis KM, Firestone MK, Lindow SE (2007). Sensitive whole-cell biosensor suitable for detecting a variety of *N*-acyl homoserine lactones in intact rhizosphere microbial communities. *Appl Environ Microbiol* **73**: 3724-3727.

DeAngelis KM, Lindow SE, Firestone MK (2008). Bacterial quorum sensing and nitrogen cycling in rhizosphere soil. *FEMS Microbiol Ecol* **in press**.

DeSantis TZ, Brodie EL, Moberg JP, Zubieta IX (2007). High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone *Microbial Ecol* **53**:371-383.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K *et al* (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72:** 5069-5072.

Fierer N, Jackson JA, Vilgalys R, Jackson R (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl Environ Microbiol* **71**: 4117-4120.

Folman LB, Postma J, Van Veen JA (2001). Ecophysiological characterization of rhizosphere bacterial communities at different root locations and plant developmental stages of cucumber grown on rockwool. *Microbial Ecol* **42:** 586-597.

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000). Rapid Method for Coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* **66**: 5488-5491.

Hawes MC, Brigham LA, Wen F, Woo HH, Zhu Z (1998). Function of root border cells in plant health: Pioneers in the rhizosphere. *Annual Review of Phytopathology* **36:** 311-327.

Hawkes CV, DeAngelis KM, Firestone MK (2007). Root interactions with soil microbial communitites and processes. In: Cardon Z and Whitbeck J (eds). *The Rhizosphere*. Elsevier: New York.

Heijnen CE, Page S, Vanelsas JD (1995). Metabolic activity of *Flavobacterium* strain P25 during starvation and after introduction into bulk soil and the rhizosphere of wheat. *FEMS Microbiol Ecol* **18:** 129-138.

Herman DJ, Johnson KK, Jaeger CH, Schwartz E, Firestone MK (2006). Root influence on nitrogen mineralization and nitrification in *Avena barbata* rhizosphere soil. *Soil Sci Soc Amer J* **70**: 1504-1511.

Hershberger KL, Barns SM, Reysenbach AL, Dawson SC, Pace NR (1996). Wide diversity of Crenarchaeota. *Nature* **384**: 420-420.

Ikenaga M, Asakawa S, Muraoka Y, Kimura M (2003). Bacterial communities associated with nodal roots of rice plants along with the growth stages: Estimation by PCR-DGGE and sequence analyses. *Soil Sci Plant Nutr* **49:** 591-602.

Jaeger CH, Lindow SE, Miller S, Clark E, Firestone MK (1999). Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and Tryptophan. *Appl Environ Micro* **65:** 2685-2690.

Kowalchuk GA, Bodelier PLE, Heilig GHJ, Stephen JR, Laanbroek HJ (1998). Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. *FEMS Microbiol Ecol* **27:** 339-350.

Loh J, Pierson EA, Pierson LS, Stacey G, Chatterjee A (2002). Quorum sensing in plant-associated bacteria. *Current Opinion in Plant Biology* **5:** 285-290.

Lubeck PS, Hansen M, Sorensen J (2000). Simultaneous detection of the establishment of seed-inoculated Pseudomonas fluorescens strain DR54 and native soil bacteria on sugar beet root surfaces using fluorescence antibody and in situ hybridization techniques. *FEMS Microbiol Ecol* **33:** 11-19.

Lynch JM, Whipps JM (1990). Substrate Flow in the Rhizosphere. *Plant and Soil* **129:** 1-10.

Macrae A, Rimmer DL, O'Donnell AG (2000). Novel bacterial diversity recovered from the rhizosphere of oilseed rape (Brassica napus) determined by the analysis of 16S ribosomal DNA. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **78:** 13-21.

McCune B, Grace JB (2002). *Analysis of Ecological Communities*. MjM Software Design: Gleneden Beach, OR.

Nunan N, Daniell TJ, Singh BK, Papert A, McNicol JW, Prosser JI (2005). Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. *Appl Environ Micro* **71:** 6784-6792.

Osborn AM, Moore ERB, Timmis KN (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol* **2:** 39-50.

Paul EA, Clark FE (1996). *Soil microbiology and biochemistry, Second edition.* Academic Press, Inc. San Diego, California.

Pett-Ridge J, Firestone MK (2005). Redox fluctuation structures microbial communities in a wet tropical soil. *Appl Environ Micro* **71:** 6998-7007.

Rozen S, Saletsky H (2000). Primer3 on the WWW for general users and for biologist programmers. In: S K and S M (eds). *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press: Totowa, NJ. pp 365-386.

Sangwan P, Kovac S, Davis KER, Sait M, Janssen PH (2005). Detection and cultivation of soil verrucomicrobia. *Appl Environ Micro* **71:** 8402-8410.

Schmidt SK, Costello EK, Nemergut DR, Cleveland CC, Reed SC, Weintraub MN *et al* (2007). Biogeochemical consequences of rapid microbial turnover and seasonal succession in soil. *Ecology* **88:** 1379-85.

Torsvik V, Ovreas L, Thingstad TF (2002). Prokaryotic diversity - Magnitude, dynamics, and controlling factors. *Science* **296:** 1064-1066.

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk HP, Schleper C (2005). Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* **7:** 1985-1995.

Ulrich A, Becker R (2006). Soil parent material is a key determinant of the bacterial community structure in arable soils. *FEMS Microbiol Ecol* **56:** 430-443.

Vandenkoornhuyse P, Mahe S, Ineson P, Staddon P (2007). Active root-inhabiting microbes identified by rapid incorporation of plant-derived carbon into RNA. *Proc Natl Acad Sci USA* **104**: 16970-16975.

Waldrop MP, Balser TC, Firestone MK (2000). Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem* **32:** 1837-1846.

Yang C-H, Crowley DE (2000). Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl Environ Microbiol* **66:** 345-351.

Yang YH, Speed T (2003). Design and analysis of comparative microarray experiments. In: Speed T (ed). *Statistical analysis of gene expression microarray data*. Chapman & Hall/CRC: Washington, D. C. pp 35-92.

Zak DR, Blackwood CB, Waldrop MP (2006). A molecular dawn for biogeochemistry. *Trends Ecol Evol* **21:** 288-295.

Figure legends

Figure 1. (A) Microcosms used in this study are constructed of plexiglass with dimensions 15cm x 5cm x 40cm. Sieved (2mm) homogenized soil is packed into the main chamber of the microcosms, and plants are grown from seedlings to maturity in the main compartment as shown. (B) After 6-8 weeks of growth, the microcosms are tipped to a 45 angle, and the solid divider separating the main chamber from the experimental chamber is replaced with a slotted divider, so that the roots will grow along the outside face of the microcosm. (C) A photograph of the microcosm shows the experimental chamber, with the roots visible; white lines circle the four soil types sampled: root tip, root hairs, mature root rhizosphere soil and bulk soil.

Figure 2. Live bacterial cell density is higher in the rhizosphere than bulk soil. Log of live bacterial cell counts were tested for normal distribution using the Shapiro-Wilk W test. Differences between root zones (treatments) were calculated using one way analysis of variance (ANOVA). The Tukey-Kramer HSD test was applied to rank the differences in magnitudes of

means; lowercase letters indicate means that are not significantly different using a p value cutoff of 0.05. For live cells, $p<0.0001$ indicating significant differences between root zones.

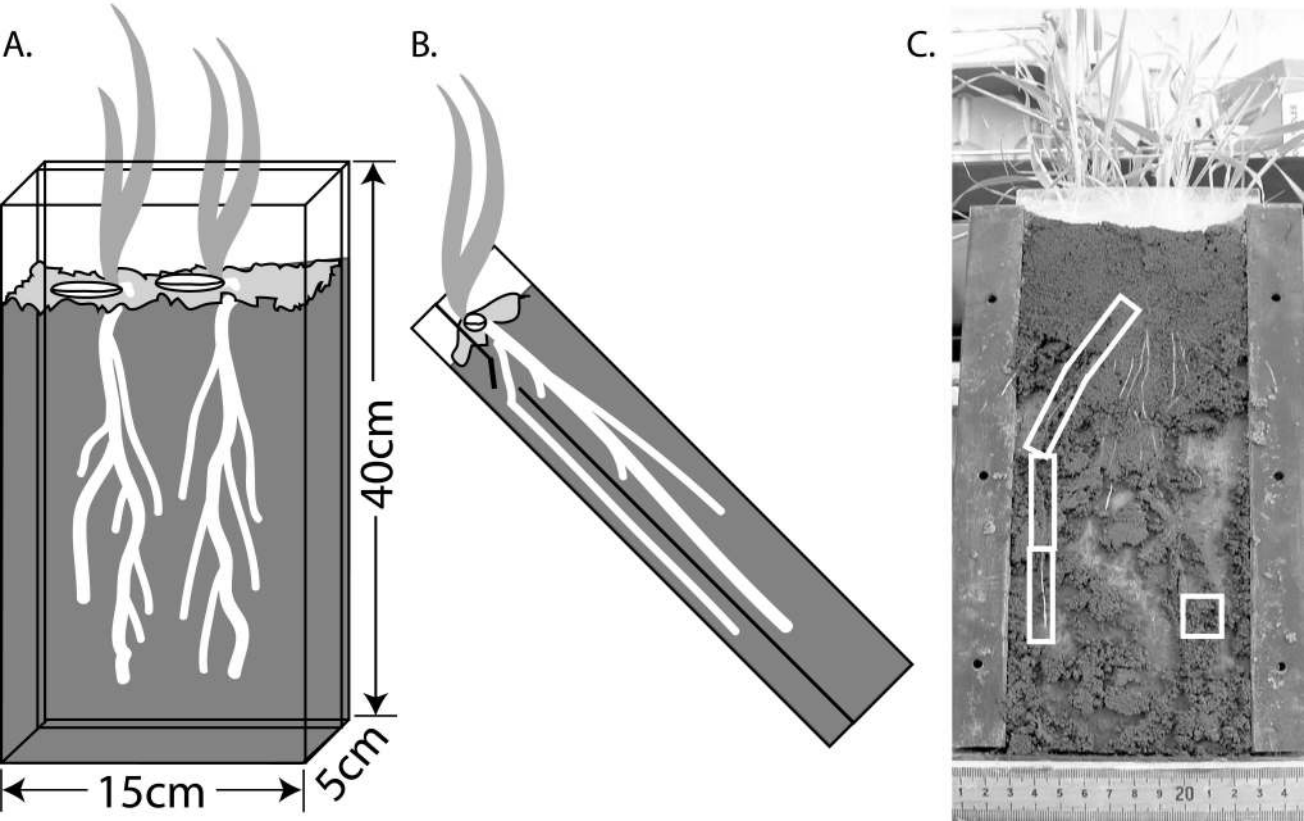
Figure 3. In (A) and (B), ordered histograms display the relative fold differences in average 16S rRNA copy numbers for each taxa in the root tip, root hairs, or mature root compared to bulk soil. The whole community (1917 taxa, A) is shown along side the dynamic community only (147 taxa, B), which are comprised of taxa with a significant ($p<0.05$) p-value in at least one of three paired t-tests comparing each rhizosphere root zone to bulk soil. The y-axis value is the fold difference in 16S rRNA copy number for each taxon, calculated as the ratio of average 16S copy number in the rhizosphere divided by the average 16S copy number in the bulk soil background. In this display, a value of one indicates that there is no fold difference over the background, and a vertical line marks this point for each root zone; a value of 2 or 3 indicates a doubling or tripling of that taxa abundance over the bulk soil. For all taxa, n=3. (C) Average hybridization scores for the most dynamic taxa from the dynamic subgroup in (B) are shown. Error bars represent standard errors, and in many cases are too small to be seen on the graph. Taxa are labeled with phyla and reference identification number, with phyla abbreviations as "Acido.", Acidobacteria; "Actino.", Actinomycetes; "Bacte.", Bacteroidetes; "Firmi.", Firmicutes; "Planc.", Planctomycetes; "Prote.", Proteobacteria; "Verru.", Verrucomicrobia.

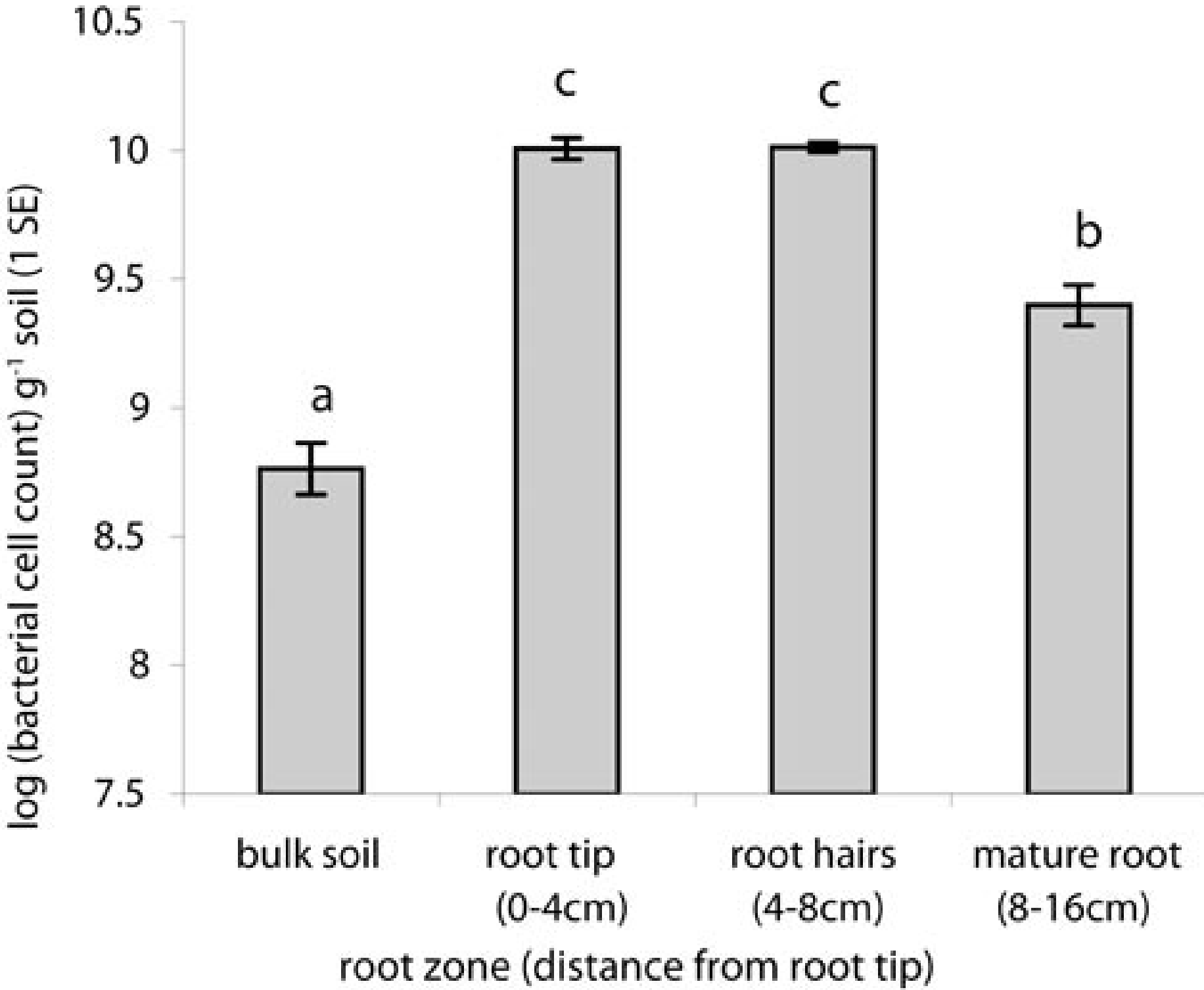
Figure 4. (A) Principle components analysis is shown for the dynamic subset of the microbial community, 147 taxa, which are the taxa that had a $p<0.05$ from one of three paired t-tests. In this analysis, principle components axes 1 and 2 explain most of the variance in the data cumulatively. Circles are drawn around the three samples that are replicates for each soil type.

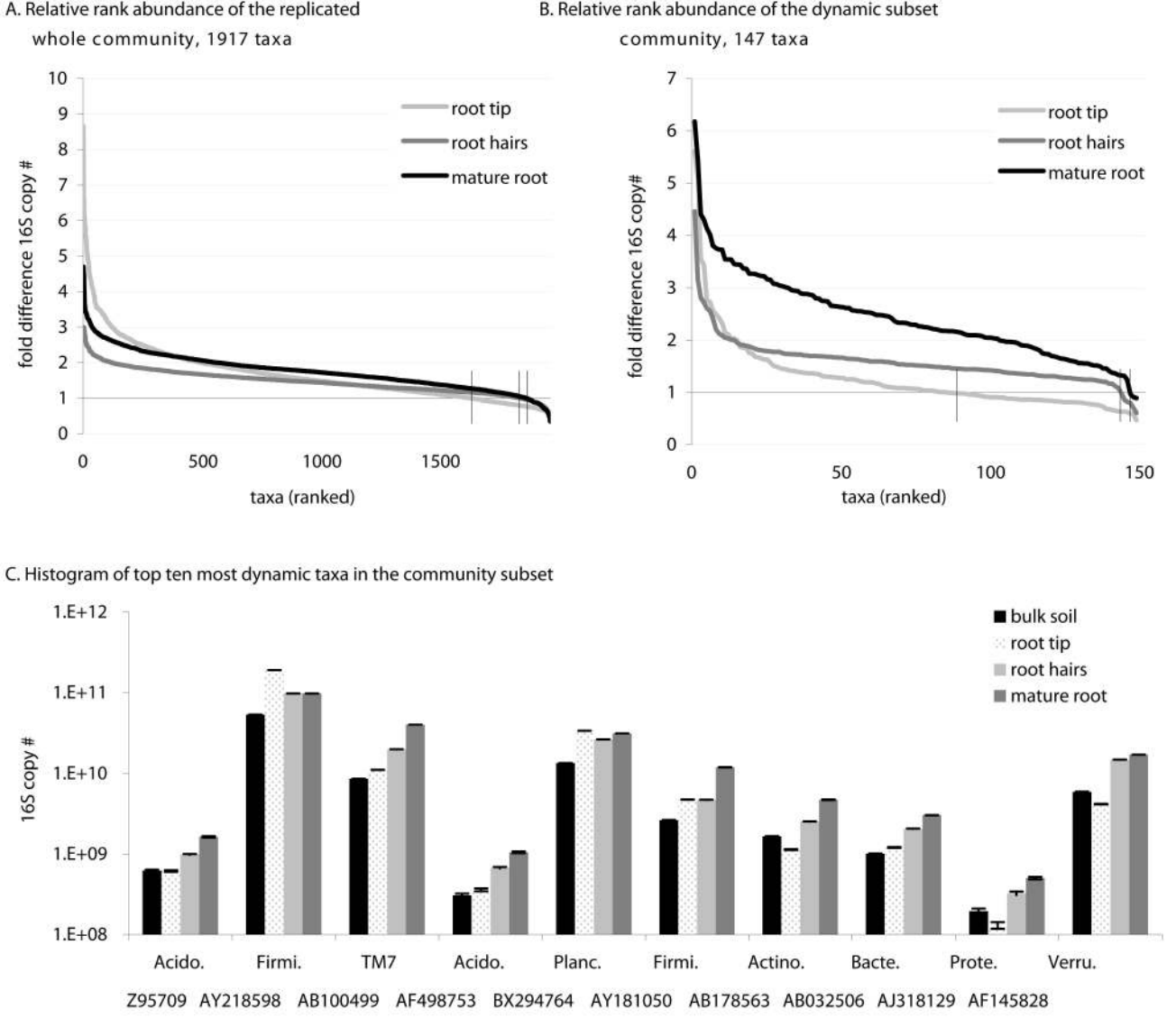
(B) Principle components analysis of twenty Archaeal taxa out of the possible 309 on the PhyloChip that were detected in our soils.

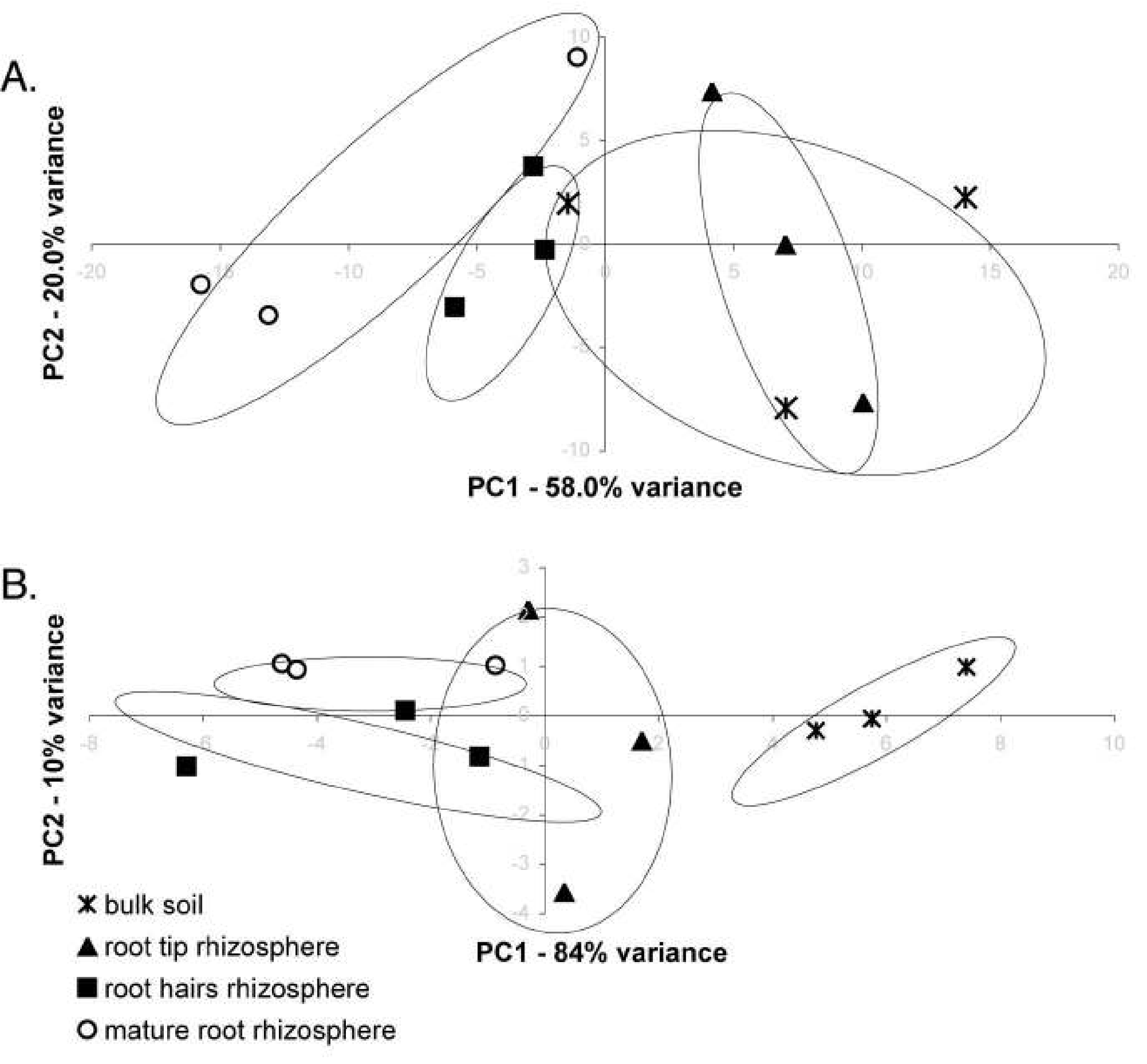
Figure 5. Relative contribution of major phyla to richness in (A) the entire community, and (B) the dynamic subset community. Richness is determined by presence or absence as defined by probe fraction; see Methods for details. Stacked bars with letters were significant by oneway ANOVA to the following p-values: (A) the entire community, Bacteroidetes p=0.0837 and All Others p=0.0650, and (B) dynamic subset, Actinobacteria p=0.049.

Figure 1




10.5
10
9.5
9
8.5
8
7.5
log (bacterial cell count) g-1 soil (1 SE)
a
c
c
b
bulk soil
root tip (0-4cm)
root hairs (4-8cm)
mature root (8-16cm)
root zone (distance from root tip)


A. Relative rank abundance of the replicated whole community, 1917 taxa
fold difference 16S copy #
taxa (ranked)
root tip
root hairs
mature root
B. Relative rank abundance of the dynamic subset community, 147 taxa
fold difference 16S copy#
taxa (ranked)
root tip
root hairs
mature root
C. Histogram of top ten most dynamic taxa in the community subset
16S copy #
bulk soil
root tip
root hairs
mature root
Acido. Z95709
Firmi. AY218598
TM7 AB100499
Acido. AF498753
Planc. BX294764
Firmi. AY181050
Actino. AB178563
Bacte. AB032506
Prote. AJ318129
Verru. AF145828


A.
PC2 - 20.0% variance
PC1 - 58.0% variance
B.
PC2 - 10% variance
PC1 - 84% variance
bulk soil
root tip rhizosphere
root hairs rhizosphere
mature root rhizosphere

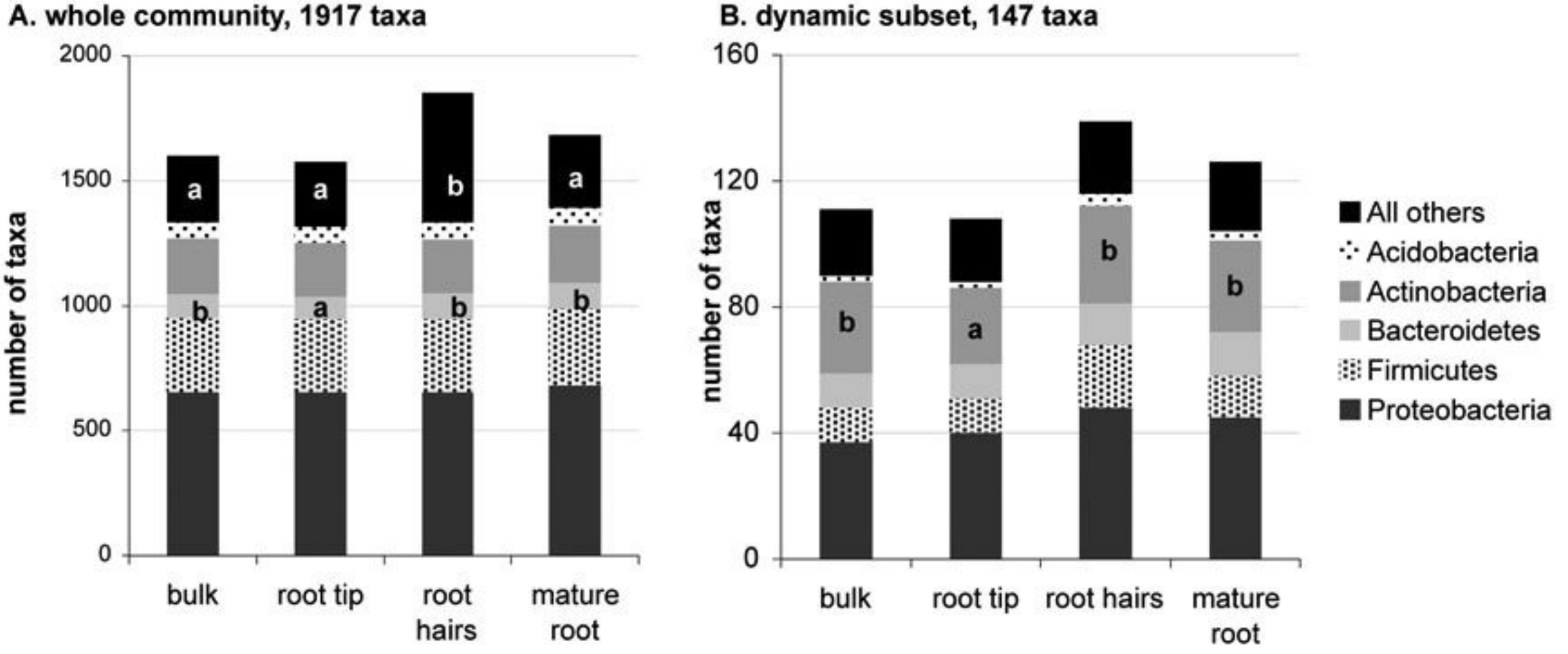

A. whole community, 1917 taxa
number of taxa
2000
1500
1000
500
0
a
a
b
a
b
a
b
b
bulk
root tip
root hairs
mature root
B. dynamic subset, 147 taxa
number of taxa
160
120
80
40
0
b
a
b
b
bulk
root tip
root hairs
mature root
All others
Acidobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria

Table 1. Primers used in this study

Primer name	target group	sequence 5'-3'	16S location	Reference
8F*	bacterial	AGAGTTTGATCCTGGCTCAG	8	Brodie, pers com
4Fa	archaeal	TCCGGTTGATCCTGCCRG	4	Hershberger *et al.*, 1996
1392R	universal	ACGGGCGGTGTGTACA	1392	Amann *et al.*, 1995
Bet680R	ß-Proteobact	TCACTGCTACACGYG	680	Fierer & Jackson 2005
Eub338F	bacterial	ACTCCTACGGGAGGCAGCAG	338	Fierer & Jackson 2005
Actino1175F	Actinobacteria	GGTACAGAGGGCTGCGATAC	1175	this study
Nitrosp1225F	Nitrospira	GGCGACACACGTGCAAC	1225	this study

Table 2. Relative contribution of taxa* to dynamic subset community by Phylum.

Phylum	Total taxa detected	Number of significant taxa in the dynamic subset	% Significant taxa in the dynamic subset of Total
Crenarchaeota	8	2	25.0
Nitrospira	5	1	20.0
Cyanobacteria	21	4	19.0
Planctomycetes	21	4	19.0
Bacteroidetes	105	19	18.1
Actinobacteria	225	38	16.9
Verrucomicrobia	33	5	15.2
Proteobacteria, δ-	35	5	14.3
Proteobacteria, α-	246	28	11.4
Proteobacteria, γ-	150	17	11.3
Acidobacteria	65	7	10.8
Proteobacteria, β-	128	12	9.4
Unclassified	34	3	8.8
Total (all Phyla)	1917	147	7.7
Firmicutes	346	24	6.9
Proteobacteria, ε-	42	2	4.8
Spirochaetes	31	1	3.2
Chloroflexi	41	1	2.4

*Only groups with more than 5 taxa are included in this table for clarity.