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Soil Microbe Active Community Composition and Capability of Responding to Litter Addition after 12 Years of No Inputs

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One explanation given for the high microbial diversity found in soils is that they contain a large inactive biomass that is able to persist in soils for long periods of time. This persistent microbial fraction may help to buffer the functionality of the soil community during times of low nutrients by providing a reservoir of specialized functions that can be reactivated when conditions improve. A study was designed to test the hypothesis: in soils lacking fresh root or detrital inputs, microbial community composition may persist relatively unchanged. Upon addition of new inputs, this community will be stimulated to grow and break down litter similarly to control soils. Soils from two of the Detrital Input and Removal Treatments (DIRT) at the H. J. Andrews Experimental Forest, the no-input and control treatment plots, were used in a microcosm experiment where Douglas-fir needles were added to soils. After 3 and 151 days of incubation, soil microbial DNA and RNA was extracted and characterized using quantitative PCR (qPCR) and 454 pyrosequencing. The abundance of 16S and 28S gene copies and RNA copies did not vary with soil type or amendment; however, treatment differences were observed in the abundance of archaeal ammonia-oxidizing *amoA* gene abundance. Analysis of ~110,000 bacterial sequences showed a significant change in the active (RNA-based) community between day 3 and day 151, but microbial composition was similar between soil types. These results show that even after 12 years of plant litter exclusion, the legacy of community composition was well buffered against a dramatic disturbance.

The high diversity of microorganisms in soil has proven challenging to investigations of community dynamics and persistence. Numerous DNA- and lipid-based investigations of soil microbial communities have suggested high spatial and seasonal variability, but these methods are often limited to the dynamics of the most dominant taxa and so leave open questions concerning persistence of rare taxa and the ability of a community to maintain key functions. Emerging evidence suggests that the majority of environmental microorganisms may be dormant (1, 2), which may account for the striking numbers of individual taxa that compose the rare biosphere. Furthermore, at least one deep sequencing effort recently revealed that microbial community membership may be stable for long time frames (3). Caporaso et al. (3) observed seasonal shifts in the abundance of dominant species but showed that all operational taxonomic units (OTUs) were present at each sampling time during a 6-year period.

There is likely a spectrum of dormancy within the soil microbial community, ranging from nonviable cells to cells that were recently active. Clearly distinguishing between active and dormant cells is complex but could be aided by the analysis of rRNA rather than rRNA genes (4). Although the dynamics of rRNA in the environment are not completely known, sporulation in *Bacillus* sp., one of the best-described mechanisms for dormancy, results in rRNA degradation immediately following sporulation that continues for a few days after sporulation initiates (5). An examination of both rRNA and rRNA genes showed phylogenetically related clusters in rRNA sequences only, suggesting that the active community might be closely related bacteria with similar ecological strategies (6). This also accounts for observations of low-abundance taxa in DNA sequence libraries found to be highly abundant in rRNA libraries (7). Focusing on the composition of rRNA may therefore be more reflective of a community that is engaged in litter decomposition and nutrient cycling.

The Detrital Input and Removal Treatments (DIRT) were established in the H. J. Andrews Experimental Forest, OR, in 1997 to test the effects of above- and below-ground plant inputs on soil organic matter stabilization. Included in the experimental manipulations is a no-input (NI) treatment where both roots and above-ground litter have been excluded from the plots. After 12 years of the field experiment, these sites provided a unique opportunity to assess the persistence of a microbial community able to use new additions of plant litter. Previous research at this location has characterized the microbial communities based on lipid (8) and DNA analyses (9) and showed that NI and control (CO) soils varied in composition after 6 years (Table 1). These investigations were likely limited to dominant OTUs and included both active and dormant microbial members. The present study examined extracted soil rRNA, characterized the community using a more intensive sequencing effort, and included a microcosm experiment where new litter additions were provided to soil microorganisms. We hypothesized that following 12 years of no above- or below-ground inputs from plants, active communities in CO and NI soils would differ, but when provided with new inputs, active communities in CO and NI soils would be more similar after a 5-month incubation.

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TABLE 1 Summary of previous findings comparing DIRT no-input and control treatments

Method of characterization	Summary of findings	Reference
Phospholipid fatty acid (PLFA)	Microbial biomass was the same across treatments. Composition was significantly different between CO and NI soils. Fungal/bacterial ratios were significantly lower in NI soils.	8
DNA-based length heterogeneity (LH) PCR	Bacterial 16S rDNA and fungal ITS compositions were significantly different between CO and NI soils.	9
DNA-based pyrosequencing	Bacterial 16S rDNA compositions differed between CO and NI (1,100 sequences). Fungal ITS compositions differed between CO and NI (475 sequences).	D. D. Myrold, unpublished data

MATERIALS AND METHODS

Sample collection. Soils were collected from the DIRT plots located in the H. J. Andrews Experimental Forest (44°15′N, 122°10′W, 531-m elevation). The DIRT plots were established in 1997 at an undisturbed, forested old-growth stand containing Douglas-fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*); they have been described previously (8, 10, 11). Soils at the site have been classified as coarse loamy mixed mesic Typic Hapludands (12). Experimental manipulations were imposed on three replicate plots, each measuring 10 m by 15 m. For the purposes of this study, soils were collected from two experimental treatments: NI and CO. The perimeter of the NI plots had been trenched to 1 m to allow the installation of an impenetrable barrier, excluding roots, and a 1-mm mesh was installed across the surface and periodically swept to prevent above-ground litter accumulation.

In August 2009, 30 soil cores (2.2-cm diameter to a depth of 10 cm) were collected from NI and CO plots. In CO plots, surface litter was removed prior to sampling, so all samples consisted only of mineral soil. Soil was sieved to 4 mm, and all cores for a given treatment were bulked, creating two composite samples. Subsamples of composited soils were extracted for DNA to establish a background gene quantity. The remaining soil was preincubated at 25°C for 10 days in accordance with standard protocols for long-term ecological reserve (LTER) sites (13).

Soil microcosms. Microcosms were established using composited soils. Each microcosm contained 30 g of soil kept at field moisture. Half of the microcosms received 0.78 g of ^{15}N-labeled Douglas-fir needles (1.25 mg C g^{-1} soil), double the rate of average field inputs (14). Before additions, the needles were leached to remove readily soluble organic compounds and chopped to mimic soil animal processing. The litter was homogeneously mixed into soil. The experiment included control/no litter (CO-NL), control/Douglas-fir (CO-DF), no input/no litter (NI-NL), and no input/Douglas-fir (NI-DF). The microcosms were sealed in glass jars to prevent drying and periodically vented to maintain aerobic conditions. Microcosms were incubated for a total of 151 days. At day 3 and day 151, four microcosms of each treatment were destructively sampled. Soil (2 g) was preserved using LifeGuard soil preservation solution (MO BIO Laboratories, Carlsbad, CA) and stored at −20°C until extraction. Due to low extraction recovery in some samples, three replicates per treatment were analyzed.

Soluble C+N and microbial biomass C+N. At each harvest time, 15 g (dry weight) soil was extracted with 50 ml of 0.05 M K_2SO_4 and shaken for 1 h before being filtered through Whatman no. 2 filter paper. Ammonium (NH_4^+) and nitrate (NO_3^-) were measured by automated colorimetry (Astoria-Pacific 300 series autoanalyzer; Clackamas, OR). Microbial biomass C and N were determined using a 24-h chloroform fumigation followed by extraction (15, 16). Combustion oxidation was used to measure total soluble C, microbial biomass C, and microbial biomass N (Shimadzu TOC-V CSH with TNM-1 module; Tokyo, Japan). A correction factor of 0.45 was used for biomass C (15), and a correction factor of 0.54 was used for biomass N (16).

Quantitative PCR. Soil RNA and DNA were extracted from each sample using the RNA PowerSoil total RNA isolation kit and the RNA PowerSoil DNA elution accessory kit (MO BIO Laboratories, Carlsbad, CA), according to the manufacturer's instructions. RNA extracts were treated with DNase I (5Prime Inc., Gaithersburg, MD) and then checked by PCR amplification to ensure complete removal of DNA. RNA extracts were reverse transcribed using SuperScript III first-strand synthesis system with random hexamers (Life Technologies, Grand Island, NY).

Both cDNA and DNA were quantified using a Qubit 1.0 fluorometer (Life Technologies, Grand Island, NY). cDNA and DNA extracts were diluted to 1.25 ng μl^{-1} in preparation for quantitative PCR (qPCR). Brilliant SYBR green qPCR master mix (Stratagene, La Jolla, CA) was used with an ABI 7500 sequence detection system (Life Technologies, Grand Island, NY). Bacterial 16S gene copy numbers were determined using primers Eub338/Eub518, according to published protocols (17, 18). Fungal 28S gene copy numbers were determined using primers LR3/LROR (19) with the following settings: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min. Data were collected at 72°C. Ammonia-oxidizing bacteria (AOB) were quantified using primers *amoA*-1F/*amoA*-2R (20) and ammonia-oxidizing archaea (AOA) were quantified using primers Arch-*amoA*F and Arch-*amoA*R (21, 22). Plasmids containing primer-specific inserts were used as standards for all assays, except AOB, which were quantified using dilutions of *Nitrosomonas europea* genomic DNA. Efficiency for all PCR runs were 95 to 105%, and standard curves had an r^2 value of >0.98. Disassociation curves were run to ensure specific amplification.

Barcoded pyrosequencing of the 16S and 28S rRNA genes. PCR was performed using fusion primers containing Titanium A or B adaptors (Roche, Basel, Switzerland), a sample-specific barcode, and gene-specific primers. Bacterial and archaeal 16S genes were amplified using F515/R806 (23) targeting the V4 hypervariable region, and fungal 28S was amplified using LR3/LROR (19) targeting regions D1 and D2. Approximately 25 ng of cDNA was amplified using Invitrogen Platinum *Taq* (Life Technologies, Grand Island, NY) with the following reagent concentrations: 1× buffer, 2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.4 μM each primer, and 0.5 U of Taq. Amplifications were cleaned using Agencourt AMPure XP beads (Beckman Coulter Inc., Danvers, MA), by following the Roche 454 Technical Bulletin 2011-002. Prior to pooling, each sample was quantified using the Qubit fluorometer to ensure that equimolar amounts of each sample would be added into a single sequencing reaction. A single pooled sample was submitted to the Oregon State University Center for Genome Research and Biocomputing and analyzed using an FLX Junior (Roche, Basel, Switzerland).

Sequence and statistical analysis. Univariate data, including qPCR and OTU abundance, were analyzed by three-way analysis of variance (ANOVA) with pairwise comparisons calculated by Tukey's honestly significant difference (HSD) test (P values of <0.05 were considered significant). Univariate statistics were analyzed using JMP version 10.0 (SAS Institute Inc., Cary, NC). qPCR data were log transformed to achieve normality. All sequence analysis was conducted using the QIIME pipeline (24). The sequence library was first separated into 16S and 28S rRNA sequences and then demultiplexed. After quality filtering, the rRNA sequence library contained 108,315 16S sequences and 2,523 28S sequences. (The low number of 28S sequences was likely a result of preferential emulsion PCR of the shorter 16S target compared to the 28S.) OTUs were identified at 97% sequence similarity using Silva 108 (25). Pairwise distances were calculated using UniFrac (26), and these data were visualized using principal coordinate plots (PCoA). The robustness of the sequencing effort was investigated by jackknife analysis. OTU tables were further

TABLE 2 Concentrations of total soluble C, total soluble N, microbial biomass C, microbial biomass N, and extracted DNA for each treatment

Treatment	Harvest day	Concn[a] Soluble C (mg C kg^{-1} soil)	Soluble mineral N (mg N kg^{-1} soil)	Microbial biomass C (mg C kg^{-1} soil)	Microbial biomass N (mg N kg^{-1} soil)	Total DNA extracted ($\mu g\ g^{-1}$ soil)
CO-NL	3	38.0 A	3.7 C	379 AB	31 A	7.6 A
	151	27.4 AB	40.2 B	304 BC	28 B	4.3 A
CO-DF	3	29.3 A	3.0 C	427 A	33 A	6.5 A
	151	26.9 AB	33.4 B	293 C	26 B	2.9 A
NI-NL	3	26.3 B	6.4 C	349 AB	33 A	3.0 A
	151	29.4 AB	74.8 A	326 BC	25 B	3.0 A
NI-DF	3	22.4 B	5.5 C	406 A	35 A	2.6 A
	151	26.0 AB	47.8 B	288 C	21 B	2.2 A

[a] Letters show Tukey's HSD comparisons ($P < 0.05$).

analyzed with multiresponse permutation procedures (MRPP) to test for significant differences among treatments (PC-ORD version 6; Gleneden Beach, OR).

RESULTS

Soluble C+N and microbial biomass C+N. Soluble C was significantly different in CO (34 mg kg^{-1} soil) and NI (24 mg kg^{-1} soil) soils at day 3 ($P = 0.02$) but did not differ at day 151 (Table 2). Soluble mineral N (the sum of NH_4^+ and NO_3^--N) was significantly different among soil types ($P = 0.001$) and litter addition ($P = 0.03$), but both parameters had significant statistical interactions with sampling day due to the large observed increased in mineral N over the 151-day incubation. Mineral N concentration was highest in the NI-NL incubations at day 151 (74.8 mg kg^{-1} soil).

Microbial biomass C significantly decreased from day 3 to day 151 (Table 2). The most dramatic decrease occurred in soils amended with Douglas-fir needles, where day 3 Douglas-Fir treatments averaged 417 mg C kg^{-1} soil and at day 151 averaged 290 mg C kg^{-1} soil. A similar trend was observed in microbial biomass N ($P = 0.002$), with an average decrease of 8 mg N kg^{-1} soil from day 3 to day 151. In addition to the chloroform fumigation determination of microbial C and N, total extracted DNA was also compared. There were no statistical differences in total DNA among plot, litter, or time; CO soils at day 3 tended to contain more DNA, but yields were variable among extractions.

Gene abundance for bacteria, fungi, and ammonia oxidizers. Bacterial 16S ribosomal gene copy numbers were measured in soils immediately after sieving but did not differ between CO (2.12×10^9 copies g^{-1} soil) and NI (1.55×10^9 copies g^{-1} soil). Soils from the incubation experiment averaged 1.3×10^9 copies g^{-1} soil and also did not differ among soil type, litter addition, or sampling time. Bacterial 16S rRNA copy numbers averaged 3.2×10^9 copies g^{-1} soil, significantly more than DNA. The rRNA copy numbers did not vary by soil type, treatment, or time, but a significant interaction among the three main factors was observed. CO-DF at day 151 did differ from NI-NL at day 3, with all other treatments intermediate (Fig. 1).

No differences between soil types or litter additions were observed in 28S gene copy numbers. 28S gene copy numbers tended to be lower at day 151 than at day 3, but this was also not significant ($P = 0.1$). Gene copy numbers were an order of magnitude lower in incubated soils than in extractions of fresh soil, however (Fig. 1; $P < 0.001$). Gene copy numbers averaged 1.8×10^7 copies g^{-1} soil, and RNA copy numbers were significantly lower, with an average of 4.2×10^5 copies g^{-1} soil (Fig. 1). The 28S RNA copy numbers were significantly lower in CO-NL at day 3 than in all other treatments, but other treatments were similar to each other.

Soluble mineral N was highest in NI-NL treatments at day 151 compared to that in all treatments (Table 2); this was mostly due to high concentrations of NO_3^- (data not shown). These data suggest the presence of an ammonia-oxidizing population that was confirmed with qPCR. AOA *amoA* gene copy numbers averaged 3.3×10^8 copies g^{-1} soil, significantly higher than AOB *amoA* gene copy numbers (8.0×10^3 copies g^{-1} soil). AOB *amoA* gene copy numbers did not differ among incubations or sampling time ($P = 0.2$). AOB *amoA* transcripts could be detected in some extracts, but amplification was not consistent in the assay. AOA *amoA* gene copy numbers were more abundant in NI than in CO soils (2.5×10^8 copies g^{-1} soil and 1.5×10^7 copies g^{-1} soil, respectively; $P < 0.001$). AOA *amoA* gene copy numbers were also higher at day 151 ($P = 0.001$) than at day 3. Similar to AOB *amoA* transcripts, AOA *amoA* transcripts did not consistently amplify.

Bacterial and fungal community composition. Bacterial community compositions based on 16S rRNA were significantly different between day 3 and day 151 for all treatments (Fig. 2; MRPP, $P < 0.001$). Each sample was represented by an average of 5,240 sequences or ~15,000 sequences per treatment. Day 3 samples did not vary based on soil or litter addition, but at day 151, CO and NI bacterial composition tended to be different (MRPP, $P = 0.06$). Pairwise comparisons revealed that CO-DF significantly differed from NI-NL at day 151 (Fig. 2; MRPP, $P = 0.03$). Jackknife analysis resulted in little variation within each sample, suggesting that these patterns were robust (data not shown).

At a ≥99% sequence similarity, 483 OTUs were identified across all treatments, with 85 OTUs found only once and 337 occurring at least twice within replicate samples. When the 337 OTUs were each analyzed for treatment effects, 68 OTUs varied between day 3 and day 151. These OTUs included putatively identified *Chthoniobacterales* Da101 and an uncultured *Xanthomonadales* (Fig. 3B). Indicator species analysis identified ~50 OTUs that differed between CO and NI soils at day 151, most of which were present in both soils but varied in percent abundance and matched those identified by univariate analysis. Indicator species analysis could not be used to identify OTUs varying between plot

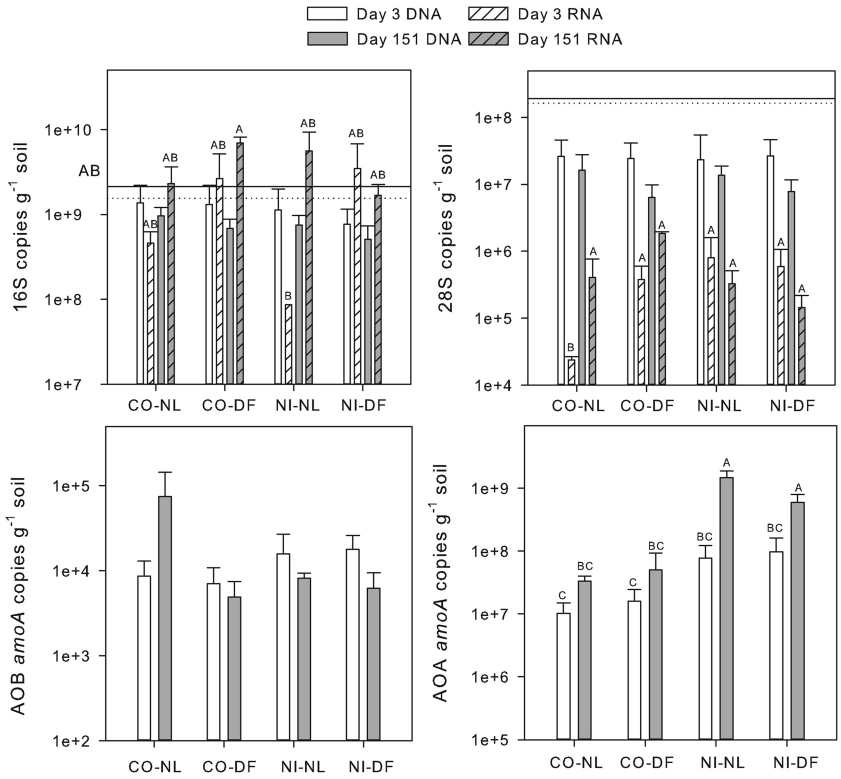

FIG 1 Gene abundance and ribosomal abundance for bacteria (16S), fungi (28S), ammonia-oxidizing bacteria (AOB), and ammonia-oxidizing archaea (AOA). Lines on the 16S and 28S graphs indicate the gene copy numbers in freshly sieved control (solid line) and no-input (dotted line) soils. Dashed lines in the AOB and AOA graphs show the assay detection limits (note that all *amoA* mRNA values were below detection). Treatments include control/no litter (CO-NL), control/Douglas-fir (CO-DF), no input/no litter (NI-NL), and no input/Douglas-fir (NI-DF). Bars represent treatment means ($n = 3$) with standard errors. Letters indicate significant treatment effects ($P < 0.05$).

or litter type, because they were not statistically different overall. Univariate analysis revealed 15 OTUs that varied between plot, and only six varied between litter treatments (data not shown).

Acidobacteria was the most common phylum across all treatments; percent abundance did not vary among treatments (Fig. 3A). There were 35 OTUs putatively identified within the *Acidobacteria*, representing groups 1, 3, and 4. The most common OTU was *Acidobacteria* Da052 (Fig. 3B).

The phylum *Proteobacteria* was the second most abundant across all treatments and at the phylum level did not vary among treatments. No significant treatment effects were present at the class or order level. The most abundant OTUs were matched to

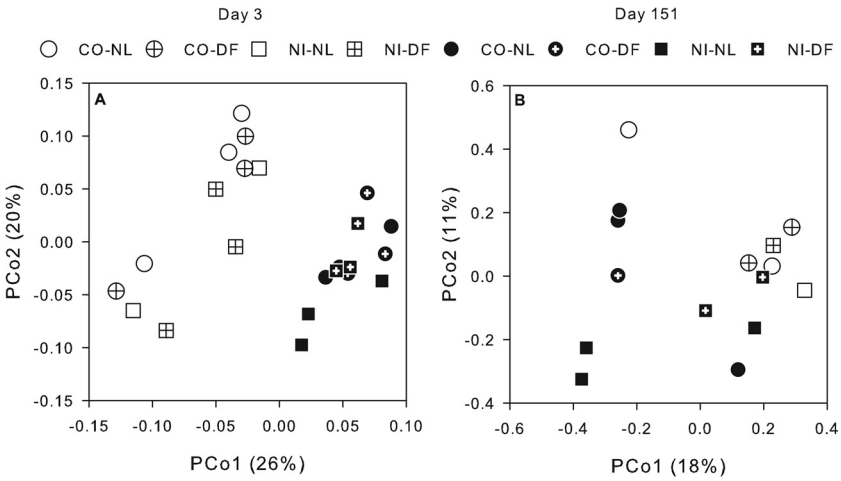

FIG 2 Unifrac-weighted principal coordinate analysis (PCoA) ordinations of the 16S (A) and 28S (B) rRNA genes. Each point represents the average distance for an individual incubation. Percentages are the amount of data explained along each axis.

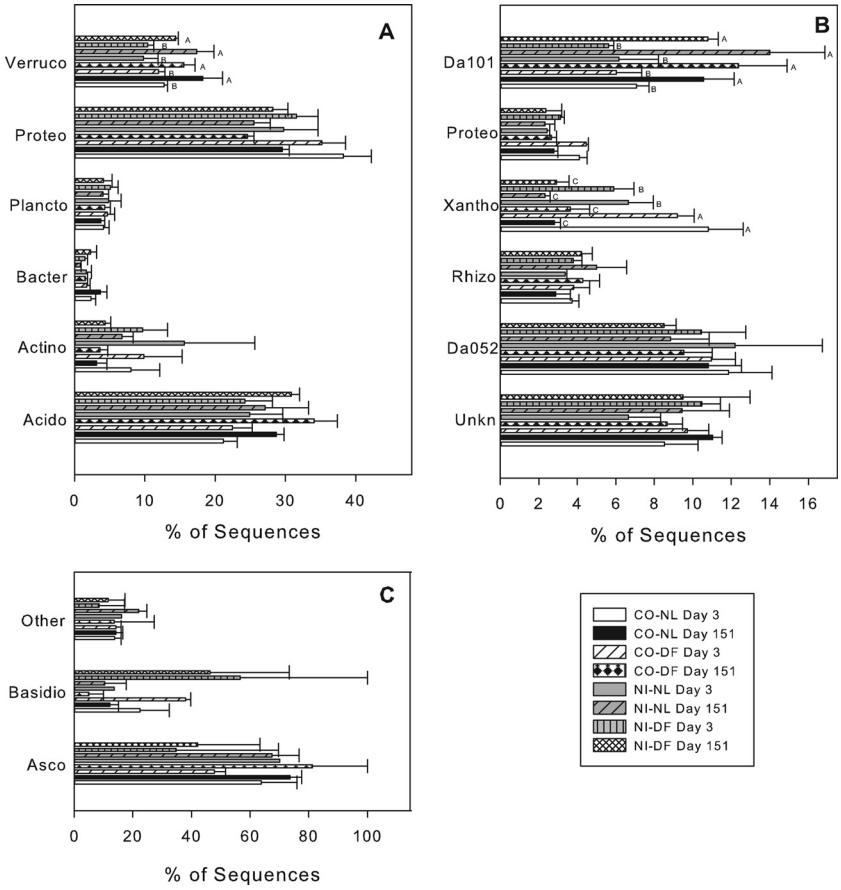

FIG 3 Percent of sequencing matching putatively identified OTUs. (A) The six most abundant bacteria phyla: *Acidobacteria* (Acido), *Actinobacteria* (Actino), *Bacteroidetes* (Bacto), *Planctomycetes* (Plancto), *Proteobacteria* (Proteo), *Verrucomicrobia* (Verruco); (B) the six most abundant bacterial OTUs at 99% sequence similarity: unknown phyla (Unkn), *Acidobacteria* Da052 (Da052), *Rhizobiales* unknown family (Rhiz), *Xanthomonadales* (Xantho), *Proteobacteria* unknown class (Proteo), *Chthoniobacterales* Da101 (Da101); (C) the distribution of *Ascomycota* (Asco), *Basidiomycota* (Basidio), and other fungi. Treatments include control/no litter (CO-NL), control/Douglas-fir (CO-DF), no input/no litter (NI-NL), and no input/Douglas-fir (NI-DF). Bars represent treatment means ($n = 3$) with standard errors. Letters indicate significant treatment effects.

Alphaproteobacteria, with a number of OTUs putatively identified as *Rhizobiales*, including the OTU shown in Fig. 3B. Eight OTUs were putatively identified as the *Gammaproteobacteria Xanthomonadales.* The most abundant *Xanthomonadales* OTU was more abundant at day 3 than at day 151 (Fig. 3B; $P < 0.001$). At day 3, the same OTU was more abundant in CO than in NI soils ($P = 0.009$).

Of the six most abundant bacterial phyla, only *Verrucomicrobia* differed among treatments (Fig. 3A). The *Verrucomicrobia* were more abundant at day 151 ($P = 0.006$). The most abundant *Verrucomicrobia* OTU, putatively identified as *Chthoniobacterales* Da101, was the only *Verrucomicrobia* OTU that significantly differed, increasing from day 3 to day 151 ($P < 0.001$).

An average of 132 fungal 28S rRNA gene sequences were analyzed per incubation, with 10 of the samples having too few rRNA sequences for further analysis. Fewer sequences and a higher variability measured by jackknife analysis (data not shown) made it difficult to observe consistent patterns. The fungal composition did, however, significantly differ between day 3 and day 151 (Fig. 2B; MRPP, $P = 0.03$). All sequences matched fungi, but targeting the D1 and D2 regions of the 28S rRNA limited the putative sequencing identification to the phylum level. Putatively identified *Ascomycota* were most abundant in all treatments (Fig. 3C), with *Basidiomycota* OTUs comprising most of the other sequences (Fig. 3C). No significant differences were seen in the phylum abundances among treatments.

DISCUSSION

Our original hypothesis that following 12 years of no above- or below-ground inputs from plants, active communities in CO and NI soils would differ, but when provided with new inputs, active communities in CO and NI soils would be more similar after a 5-month incubation, was not supported. Litter addition had no observed effect on the quantity or composition of the bacterial or fungal community, and in fact CO and NI soils were surprisingly similar regardless of additions. Brant et al. (8) did not observe differences in total microbial biomass between CO and NI based on phospholipid fatty acid (PLFA) analysis (Table 1). Microbial C and N as determined by chloroform fumigation agreed with these earlier findings (Table 2). Even after 12 years, CO and NI soils support similar microbial biomass.

There are several possible explanations for these findings: (i) the high concentration of C stored in the soils, (ii) the low concentration of fresh C input added to incubations, (iii) preleaching

of litter to remove the soluble fraction, and (iv) standard soil handling procedures.

This old-growth coniferous soil ecosystem, with andic soils, may have an increased capacity to store C compared to soils of other mineralogy. Control soils contained 56.4 g C kg^{-1} soil, and after 12 years, NI soils still contained 40.4 g C kg^{-1} soil (27). The large reserve of soil C in NI soils may continue to sustain an active decomposer community. We are currently following up with similar experiments in soils that differ in C content to understand how C storage may impact microbial persistence.

To mimic double the rate of yearly input, 1.25 mg C kg^{-1} soil was added in the form of preleached litter. These additions did not substantially change the size of the soluble C pool (Table 2) and presumably by removing the soluble fraction did not stimulate a community that would take advantage of readily available, small molecular weight molecules. It is worth noting, however, that DF additions did result in higher minimal bactericidal concentrations (MBC) at day 3 in both soil types, but in both cases MBC had significantly decreased at day 151 (Table 2).

Soils used in incubations were collected and handled in accordance to standard soil handling procedures at LTER locations (13). These guidelines suggest preincubating soils at constant moisture to stabilize the soil microbial biomass. Peterson and Klug (28) examined the effects of soil handling (sieving, incubation, and temperature) on microbial communities using PLFAs. Sieving and incubation resulted in limited changes in PLFA profiles, but a decrease in the fungal marker 18:2ω6,9 was observed. Similarly, 28S rRNA gene copy numbers significantly decreased in incubated soils at day 3 compared to freshly sieved soils (Fig. 1). The mechanism for decreased 28S rRNA gene copies during preincubation is not known. Forest fungal populations, dominated by ectomycorrhizal (EcM) fungi, are sensitive to changes in plant inputs (29, 30), and so simply removing the CO soils could have resulted in this decrease. Decreases were also observed in NI soils, however, so perhaps sieving destroyed hyphae. Additional soil handling may have resulted in increased competition between bacteria and fungi (31). Therefore, it remains unclear if the preincubation created artifacts that masked the effects of litter additions on CO and NI soils, but even on fresh samples CO and NI soils did not vary in 16S or 28S rRNA gene copy numbers.

Consistently low rRNA copy numbers and difficulty in amplifying 28S rRNA for sequencing suggest that few fungi were active during the incubation. Few studies have targeted soil rRNA of the fungi (32), and fewer still have included sequencing to determine the identity of OTUs (7). Given the important roles of EcM fungi (belonging primarily to the *Basidiomycota*), we expected to observe higher proportions of these fungi in the CO soils, but the abundance of *Basidiomycota* sequences did not vary among treatments. The lack of relative abundance differences, within the fungi and most of the bacteria, may be due to the methodological constraints of 454 sequencing (33). Recent studies have observed that 454 sequencing data are not quantitative (33) and that low sampling depth may result in high within-replicate variability (34). Variability in the abundance of fungal sequences was higher than bacterial sequences, and sequencing depth was much lower.

Forty percent of bacterial sequences matched one of six OTU groupings (Fig. 3B). The dominance of so few organisms in the rRNA sequence library was somewhat surprising, given previous estimates of diversity in soils from these forest types using DNA-based clone libraries (17). Baldrian et al. (7) reported decreased diversity and evenness in rRNA compared to those in rRNA genes in a forest soil; this observation could account for the patterns observed in H. J. Andrews soils. These comparisons are also consistent with the notion of a small subpopulation of active microorganisms (1, 6).

Bacterial composition did not vary at day 3; by day 151, active bacteria in NI-NL soils were significantly different from other treatments. No clear patterns in bacterial sequence abundance emerged that were consistent with the total community analysis. Sequences were analyzed both with (Fig. 2) and without (data not shown) taxonomic weighting. No differences were detected in the unweighted analysis, similar to the observations of Lemos et al. (35). These results point to the potential importance of taxonomic relatedness to fulfill similar ecological niches (6).

Verrucomicrobia was the only phylum that significantly differed among treatments, decreasing from day 3 to day 151 (Fig. 3A). This was due primarily to differences in *Chthoniobacterales* Da101 (Fig. 3B). *Chthoniobacterales* is one of three described orders in the phylum *Verrucomicrobia*. Most members of the order appear to be soil-inhabiting bacteria, but some are endosymbionts of nematodes (36). The type strain *Chthoniobacter flavus* is a chemoheterotroph capable of growing on a variety of polymeric substances, at pH 4 to 7, and has a low growth rate, requiring 3 months between culture transfers (36).

The representative sequences *Chthoniobacter* Da101 and *Acidobacteria* Da052 were both recovered as rRNA from Dutch pasture soils (37, 38). Although the ubiquity of members of the *Acidobacteria* and *Verrucomicrobia* across soils is well known, the high similarity of sequences in our study to those also recovered during rRNA analysis in Dutch soils may point to a more narrowly defined collection of ubiquitous soil bacteria that are active most of the time. Like *Chthoniobacter*, most cultured *Acidobacteria* are slow-growing aerobic chemoheterotrophs (39, 40). A meta-analysis of several DNA-based studies also pointed to higher relative abundance of *Verrucomicrobia* and *Acidobacteria* in bulk soils than in rhizosphere soils (41).

Although DNA clone libraries at a nearby site identified several OTUs putatively matched to *Bradyrhizobium* (member of the *Alphaproteobacteria*) (17, 42), current sequence libraries contained <10 sequences matching to this genus. These results highlight the need to further investigate microbial communities based on their active or recently active members. The OTU putatively identified as an uncultured *Xanthomonadales* was more abundant in CO than in NI soils at day 3 and decreased in all treatments at day 151. Although the putatively identified sequences matched an uncultured organism, other members of *Xanthomonadales* are well known plant pathogens infecting conifers, among other plant types (43).

Although few differences were observed between the general bacterial and fungal communities, AOA data suggest that the ability to persist and fulfill defined ecological niches may be highly variable between taxa. AOA abundance was highest in NI soils (Fig. 1) but was several orders of magnitude higher than AOBs in both soil types. In a previous study, AOAs were not detected at a nearby location (20), but this result could be due to the use in the previous study of an early AOA primer pair that has since been redesigned based on a quickly expanding sequence collection (22). More research is needed to understand the role of AOAs in forest soils. The high abundance of AOA gene copies in NI soils was consistent with studies that have shown increased AOA popula-

tions in microcosms containing low NH_4^+ (≤20 mg NH_4^+-N kg^{-1} soil) (44). For comparison, NI and CO soils averaged 0.9 mg NH_4^+-N kg^{-1} soil. Although mRNA could not be detected, the increase in gene abundance suggests that AOAs were reproducing under these conditions and resulted in increased mineral N (Table 2). No attempt was made in this study to remove rRNA or use *amoA* primers during reverse transcription, strategies that may enrich for transcript. Griffiths et al. (45) reported higher AOA abundance in fallow versus cropped soils. These trends suggest a plant-microbe interaction that warrants further investigation.

The legacy of plant inputs that contribute to high soil OM in this ecosystem continued to shape the active microbial community even a decade after alteration. In spite of 12 years of soils not receiving plant inputs, the membership of the soil microbial community had not changed, and similar populations of microorganisms were active in both CO and NI soils. It will be important to monitor these sites in the coming decades to observe if CO and NI soils eventually begin to diverge in microbial composition. This speaks to the importance of long-term study plots such as the DIRT experiment to understand microbial dynamics. Periodic microbial characterization of soils in the DIRT experiment in the future will shed light on the extent of microbial persistence.

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