# Afforestation Alters the Composition of Functional Genes in Soil and Biogeochemical Processes in South American Grasslands[▽][†]

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Soil microbes are highly diverse and control most soil biogeochemical reactions. We examined how microbial functional genes and biogeochemical pools responded to the altered chemical inputs accompanying land use change. We examined paired native grasslands and adjacent *Eucalyptus* plantations (previously grassland) in Uruguay, a region that lacked forests before European settlement. Along with measurements of soil carbon, nitrogen, and bacterial diversity, we analyzed functional genes using the GeoChip 2.0 microarray, which simultaneously quantified several thousand genes involved in soil carbon and nitrogen cycling. Plantations and grassland differed significantly in functional gene profiles, bacterial diversity, and biogeochemical pool sizes. Most grassland profiles were similar, but plantation profiles generally differed from those of grasslands due to differences in functional gene abundance across diverse taxa. Eucalypts decreased ammonification and N fixation functional genes by 11% and 7.9% ($P < 0.01$), which correlated with decreased microbial biomass N and more $NH_4^+$ in plantation soils. Chitinase abundance decreased 7.8% in plantations compared to levels in grassland ($P = 0.017$), and C polymer-degrading genes decreased by 1.5% overall ($P < 0.05$), which likely contributed to 54% ($P < 0.05$) more C in undecomposed extractable soil pools and 27% less microbial C ($P < 0.01$) in plantation soils. In general, afforestation altered the abundance of many microbial functional genes, corresponding with changes in soil biogeochemistry, in part through altered abundance of overall functional gene types rather than simply through changes in specific taxa. Such changes in microbial functional genes correspond with altered C and N storage and have implications for long-term productivity in these soils.

Although soil microbes mediate nearly all biogeochemical cycles in terrestrial ecosystems, we understand very little about how environmental changes affect these microbes and their functions (14, 17, 20, 29, 37). Studying how environmental changes affect microbial functioning should help in predicting how biogeochemical cycles will respond to changing climate and land use more broadly. Because of high levels of apparent redundancy for many biogeochemical gene families across microbial groups (9), small-scale environmental effects that alter some microbial groups will not necessarily alter overall biogeochemical functioning (20). In contrast, large-scale environmental perturbations, such as land use change, could drive major shifts in microbial populations that result in substantial biogeochemical changes. The goal of this study was to examine how soil microbes and their associated biogeochemical functions responded to a land transformation, the conversion of native grassland to forest plantations.

Many studies have examined the effects of changing land use on soil microbial diversity; however, shifts in diversity will not necessarily alter the ability of soil microbes to perform biogeochemical functions (8, 26). Functional gene microarrays that compare many orthologous gene sets controlling biogeochemi-

cal functions have recently emerged as a way to examine genes across a broad range of microorganisms and functions simultaneously (23, 27). The Geochip microarray (version 2.0) simultaneously quantifies several thousand genes from diverse species and groups involved in soil C and N cycling (27, 50, 51). This array allows for a detailed analysis of the biogeochemical gene profiles of soil microbes and is ideal for understanding how profiles change in response to environmental perturbations and experimentally imposed conditions.

Afforestation, defined by the Kyoto protocol as the establishment of tree plantations on land without forests for >50 years, is an ideal system for studying the effects of land use change on soil microbial functions. Such plantations already cover 140 million ha globally, with 2.8 million ha afforested annually; this rate is likely to increase as demand for wood products and the use of plantations as carbon offsets grows (15). Afforestation has many impacts on soil C and nutrient cycling that are likely linked to microbial activity and functions (3, 25). Studying the impacts of this land use change on soils should help improve predictions of plantation productivity and the current and future capacity of these systems to sequester carbon.

Plantation productivity and potential C sequestration are linked to microbial C and N cycling in soils. For instance, microbial decomposition of vegetation inputs to soils controls the N supply available to plants (36, 45). Since N is the soil nutrient most commonly limiting plant growth, changes in soil N cycling will often influence plant nutrient uptake and productivity (2, 45). In turn, alterations in nutrient uptake by

plants feed back into the quantity and quality of plant material supplied to microbial processes. Hence, changes in soil microbial functional genes and activity could alter the long-term carbon sequestration potential of plantations.

In this study, we examined the effects of *Eucalyptus* plantations in comparison to native grasslands in Uruguay. *Eucalyptus* is the second most common genus used for afforestation globally and comprises ~40% of the plantation area in southern South America and Brazil (10). Species of *Eucalyptus* are desirable for their robust growth in different climates and for their resistance to drought, pests, and low-nutrient soils; *Eucalyptus* also has the highest rate of growth of any commonly used genus in warm climates (10, 21, 41). In turn, Uruguay has the fastest rate of afforestation increase in terms of percentage increase and the second greatest in total area in South America, attributable in part to government subsidies (10, 22). The fast tree growth rate and proximity to paper mills in Uruguay gives these plantations one of the highest potential economic rates of return for forest products in the Western Hemisphere (9). Uruguay is a particularly good location for studying the effects of *Eucalyptus* plantations due to many replicated sites with similar planting techniques, similar forest age classes, and soil substrates. Together with the historical absence of trees in the Rio de la Plata grassland of Uruguay and Argentina, the region is an ideal one for studying the effects of land use change on soils (30, 31).

Because *Eucalyptus* plantations are common globally and tend to produce lower-quality substrates for microbes than grasses do (40, 49), we hypothesized that these lower-quality substrates (e.g., higher phenolic and other secondary compound content and higher C:N ratio) would alter the functional gene composition and diversity of microbial communities. These changes would in turn lead to altered storage of nutrients in soil pools, particularly pool sizes of soil carbon and nitrogen.

## MATERIALS AND METHODS

**Site description and soil sampling.** We studied eight sites with adjacent plots of native grasslands and *Eucalyptus* plantations. The sites ranged from 50 to 200 m in elevation and were located near Minas, the capital of the Lavalleja department of Uruguay (34.37°S, 55.23°W). Mean annual precipitation was 1,150 mm, and the mean annual temperature was 16.5°C. Soils of the region are Udolls, Udalfs, and Udepts, developed over granitic rock; soils at our sites were mostly Hapludalfs, with a typical soil depth of 30 cm (11, 16). Native grasslands in this region are a mix of $C_3$ and $C_4$ grasses that are frequently used for cattle and sheep grazing. *Eucalyptus* plantations in our study sites were not fertilized and were established between 1992 and 1996. Three of the plantations were in their second rotation, and five were original plantings. Prior to afforestation, the plantation sites were native grasslands like their adjacent controls.

In September 2005, we sampled the top 10 cm of mineral soil using a 1.9-cm-diameter soil core. At each adjacent grassland-*Eucalyptus* pair, we collected five cores per transect along two 10-m transects at least 10 m from the edge in each vegetation (10 cores per vegetation; 20 cores per site). All cores for each vegetation type (10 cores) at each site were composited in a polyethylene bag and stored on ice for transport to the laboratory. Samples were homogenized and sieved (2 mm) to remove roots and rocks. Fresh soils were used for biogeochemical analyses; subsamples were stored at −80°C for DNA extraction.

**Soil biogeochemical analyses and statistics.** Soil total extractable carbon and nitrogen, $NH_4^+$, $NO_3^-$, and microbial biomass C and N were determined as described previously (2). The methods were modified by using 2 g of field moist soil instead of 30 g. Total air-dried soil and litter C and N were determined by combustion in a Carlo-Erba elemental analyzer (CE Elantech, Lakewood, NJ). Air-dried soil and litter $^{13}C$ was determined using a Finnigan MAT Delta Plus

XL continuous-flow mass spectrometer system; results are expressed as δ-$^{13}C$ per mille versus the Pee Dee belemnite standard.

Leaf total phenolics were extracted from air-dried litter by placing 0.02 g of ground leaf material in a centrifuge tube and adding 0.75 ml of 70:30 acetone: water. The samples were placed in a sonicating bath for 10 min and then centrifuged at 10× gravity for 2 min. The supernatant was decanted and the process was repeated on the leaf material three times to ensure that all phenolics were extracted (46). Concentrations of total phenolics in the extracts, diluted 200× with doubly deionized water, were measured using the Folin method with a calibration curve of gallic acid solution (47).

We tested for significant differences in biogeochemical variables between grassland and plantation using paired *t* tests in the SAS software program (SAS Institute, Cary, NC). All distributions were determined to be Gaussian before analysis. The level of replication was the individual site (one vegetation pair).

**DNA extraction and precipitation.** DNA was extracted from 10 g of soil using the "Powermax" kit (Mo Bio Laboratories, Carlsbad, CA). The manufacturer's instructions were followed except for two modifications that reduced DNA shearing to produce high-molecular-weight DNA ideal for whole-community genome amplification. The first modification was to grind the soil in a mortar and pestle with liquid nitrogen instead of bead beating the samples. The other modification was to place the samples in a 60°C incubator and rotate the tubes gently, 30 oscillations per minute, for 1 h.

Extracted DNA was then concentrated by alcohol precipitation. One ml of DNA extract was combined with 0.8 ml ice-cold isopropanol and 0.1 ml 3 M NaCl; the mixture was inverted to mix and chilled at −20°C for 30 min to precipitate the DNA. The samples were then centrifuged at 10,000 × *g* at 4°C for 10 min, the supernatant was removed and discarded, and the pellet containing the DNA was dried in a speed vacuum. The pellet was then washed with 70% ethanol and centrifuged at 10,000 × *g*; the supernatant was removed, and then the DNA pellet was dried by using a speed vacuum and then resuspended in 50 µl of deionized water.

**Terminal restriction fragment length polymorphism analysis.** We examined the diversity of soil bacterium rRNA genes of the sites using terminal restriction length polymorphism analysis (TRFLP), following the procedures of Fierer and Jackson and of Blackwood et al. (4, 18). We modified the method by using the restriction enzyme HaeIII. Replicate fluorescently labeled PCR products were then digested for 6 h in a water bath with HaeIII and buffer as per the manufacturer's instructions (Promega, Madison, WI). Digested fragments were separated and quantified on an ABI 3700xl DNA sequencer using GeneScan software (Applied Biosystems, Foster City, CA). Analysis was computed as per the method of Fierer and Jackson (18) with distinct fragments treated as operational taxonomic units (OTUs). We calculated the Shannon diversity index (*H*) based upon OTU presence and relative abundance. We tested for differences in diversity (*H*) between vegetation types using a paired *t* test and for correlations between biogeochemical variables and *H* using Pearson's correlation coefficient.

**Whole-community genome amplification.** To obtain sufficient genomic DNA and to dilute the effect of compounds that coextract with soil DNA and inhibit microarray hybridization and signal detection, we performed a whole-community genome amplification by multiple strand displacement amplification (50). An Illustra GenomiPhi V1 DNA amplification kit (GE Healthcare Life Sciences, Piscataway, NJ) was used as described by Wu et al. (50) as per the manufacturer's instructions, with several minor modifications. Briefly, 100 ng of DNA was mixed with 9 µl of sample buffer (containing random octamer primers) and denatured in a thermocycler at 95°C for 5 min. We then added to the sample 9 µl of reaction buffer (salts and deoxynucleotide triphosphates), 1 µl enzyme mix (Phi29), 1 µl single-stranded DNA binding protein (260 ng/µl), and 1 µl spermidine solution (1 mM); the mixture was thoroughly mixed, incubated in a thermocycler at 30°C for 6 h, and then heated to 65°C for 10 min to stop the enzymatic reaction. All pipetting steps were performed in a sterile laminar flow hood to prevent the introduction of contaminant DNA prior to amplification. Nontemplate controls were amplified using sterile deionized water instead of sample DNA and were treated identically to the other samples throughout subsequent analyses.

**Microarray hybridization.** The functional gene microarrays (FGA or Geochip 2.0) used in this study were constructed as described previously (27, 28, 35). FGAs contained >27,000 gene- and group-specific oligonucleotide probes (50 bp each) that target genes essential to carbon, nitrogen, sulfur, and metal transformations and biogeochemical cycling. Three criteria were used to ensure specificity of the probes: ≤90% sequence identity to nonspecific targets, ≤20 bases of identical sequence stretches, and ≤35 kcal mol$^{-1}$ binding free energy between probes and nontarget sequences (35).

Amplified DNA was fluorescently labeled using Cy5 dye and the Klenow fragment (28). Briefly, 20 µl of amplified DNA, 20 µl of 2.5× random primers (octamers; Invitrogen), and 0.3 µl 10 mM spermidine were mixed and heated to

99.9°C for 5 min in a thermocycler and then immediately chilled on ice. To these samples we added 2.5 μl deoxynucleoside triphosphates (5 mM dATP, dGTP, dCTP, and 2.5 mM TTP), 1 μl 2.5 mM Cy5-UTP, 2 μl of 80 U Klenow (Invitrogen), 0.7 μl recombinant *Escherichia coli* RecA (490 ng/μl; Invitrogen), and 13.8 μl sterile deionized water. Samples were mixed by inverting and then centrifuged and incubated in a thermocycler at 37°C for 6 h. Once Cy5 was added, all remaining steps were conducted in low light to preserve the activity of the fluorescent dye. After incubation, the samples were cleaned using a QIAquick PCR purification kit, following the manufacturer's instructions (Qiagen, Valencia, CA). We also used buffer PB for the QIAquick kit to avoid interference from the indicator dye in the standard PBI buffer (Qiagen). Cleaned and labeled samples were dried down in a speed vacuum.

A prehybridization solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml bovine serum albumin was heated to 50°C in a water bath. Microarrays were incubated in the 50°C prehybridization solution for 45 min, then washed 3× with sterile deionized water and 1× with isopropanol, and then dried with filtered compressed air. Microarrays, hybridization chambers, lifter coverslips, pipette tips, and 5× SSC were warmed to 60°C in a hybridization oven. For each sample, 38.4 μl of hybridization mix (final concentration, 50% formamide, 5× SSC, 0.10% SDS, 0.1 μg/μl salmon sperm DNA, 0.0825 mM spermidine) was added to the dried-down sample and mixed thoroughly.

The solution was incubated at 95°C in a thermal cycler for 5 min and then kept at 60°C while 1.6 μl of 490-ng/liter RecA was added and mixed. On top of a 60°C heating block, all 40 μl of sample solution was pipetted onto the microarray at the edge of the lifter coverslip, and capillary action was used to cover the microarray surface with solution. The microarrays were sealed in watertight hybridization chambers and incubated at 50°C in a water bath overnight. Microarrays were then washed in a bath of gently shaking 50°C solution of 1× and 0.1% SDS. Without allowing the microarrays to dry, we transferred them to a gently shaking bath of the same solution at room temperature. The microarrays were transferred to a gently shaking bath of 0.1× SSC and 0.1% SDS and then through four baths of 0.1× SSC. Microarrays were then blown dry with filtered compressed air.

**Microarray processing, image analysis, and statistics.** Microarrays were scanned on a Scan Array 5000 instrument (GSI, Moorpark, CA). We used 100% laser power and adjusted the photomultiplier tube gain to maximize fluorescence while minimizing saturated probes. Microarray images were analyzed using the Imagene 6.0 software program (Biodiscovery, El Segundo, CA). To avoid false positives, each poor-quality probe and any probes with a signal-to-noise ratio less than 2 were manually excluded. The signal-to-noise ratio was calculated as (signal intensity − background)/standard deviation. Each sample was run on three replicate arrays; only probes where there was a detectable hybridization signal on at least two out of three replicates were analyzed. To normalize values across arrays, each probe's signal intensity was divided by the mean signal intensity for its array. We then calculated the mean signal intensity across the replicate slides. For each probe, we divided the signal intensity of the plantation by that of its paired grassland control and made the distribution symmetrical around 0 by taking the log base 2 (henceforth referred to as "log change").

Distributions of log change for given genes of interest were generated in SAS (SAS Institute, Cary, NC). For each gene of interest, we used the Wilcoxon signed-rank test to test if afforestation increased or decreased a given gene type (e.g., nonzero log change). The signed rank test was chosen over a one-sample *t* test because the distributions of log change for most genes of interest were not Gaussian. We report the overall effect of treatment on a category of probes as the median, since all of the distributions were not normally distributed.

We tested if the composition of functional genes differed between treatments using multivariate approaches. We used a pairwise centroid linkage hierarchical clustering algorithm from the software program Cluster (http://rana.lbl.gov) (12) and visualized the clustering results using the Treeview software program (see above URL) (12). Nonmetric multidimensional scaling (PCOrd version 5) was used to create an ordination that represented the functional gene profile in two dimensions and to calculate correlations between ordination and biogeochemical variables.

## RESULTS

Across all probes detected with the microarrays (830 total probes), afforestation altered functional gene profiles in concert with biogeochemical pool sizes. Nonmetric multidimensional scaling of all probes produced a two-dimensional ordi-
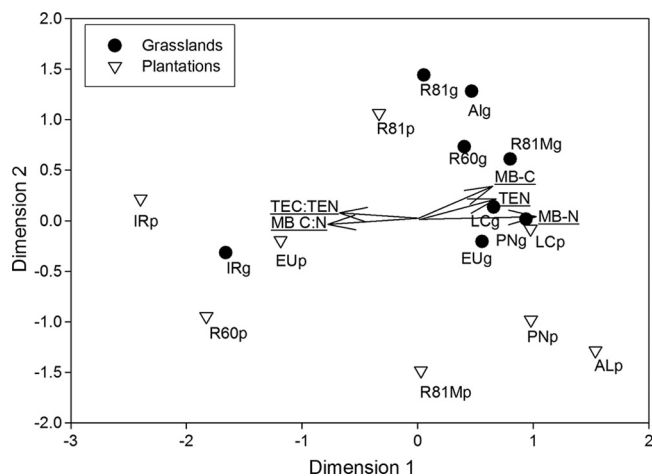


FIG. 1. Biplot of nonmetric multidimensional scaling analysis of all microarray probes (830 total). Dimensions 1 and 2 represent 69% and 27% of the variability in the original microarray data set. Arrows represent correlations between ordination dimensions (functional gene profile) and biogeochemical pools with pool names underlined. MB-C, microbial biomass carbon; MB-N, microbial biomass nitrogen; TEN, total extractable nitrogen; MB C:N, ratio of carbon to nitrogen in microbial biomass; TEC:TEN, ratio of total extractable carbon to total extractable nitrogen.

nation with axes 1 and 2 representing 69% and 27%, respectively, of the variation in composition and relative abundance of genes from the eight sites. Seven of eight grasslands had very similar functional gene profiles and grouped closely together in ordination space (Fig. 1). Two plantations had similar profiles to the grasslands, while three other plantations separated from the grasslands along dimension 2 and three other plantations separated along dimension 1. Microbial biomass C, N, and total extractable N were positively correlated with dimension 1, indicating larger pools of these C and N forms in gene profiles associated with grasslands (Fig. 1 and Table 1). The C:N ratio of microbial biomass and th C:N ratio of total extractable nutrients were negatively correlated with dimension 1, indicating narrower ratios in grassland profiles.

Like relationships among microbial C and N and soil N pools, soil bacterial diversity was strongly related to microbial C and N at our sites. The H value of soil bacteria was positively correlated with microbial biomass C ($r = 0.53$; $P = 0.03$) and microbial biomass N ($r = 0.58$; $P = 0.02$) (see Fig. S1 in the supplemental material). Total extractable C was negatively correlated with H ($r = -0.55$; $P = 0.03$) (see Fig. S1 in the supplemental material). Though still relatively diverse, afforested plots also had lower average diversity ($H = 2.6$) than grassland ($H = 2.9$; $P < 0.01$) (Table 1). Diversity ranged widely across sites and vegetation types (see Fig. S1 in the supplemental material) (Table 1).

Afforestation also altered the abundance and composition of carbon degradation genes in soils (Table 2; see Fig. 3). Cluster analysis revealed several groups of carbon degradation probes (groups 1 and 3) with higher relative abundancea in plantations (see Fig. 3). There were also several probes (group 2) that showed little difference in pattern between vegetation types (see Fig. 3). These unchanged probes suggest there are microbial strains whose functional genes are insensitive to at least

TABLE 1. Soil biogeochemical properties

| Soil or litter property | Value for vegetation[a] | |
| --- | --- | --- |
| | Native grassland | Eucalypt plantation |
| **Litter** | | |
| % C | 44 (0.38) | 53 (0.23)*** |
| % N | 0.55 (0.05) | 0.68 (0.02) |
| Phenolics (% of dry leaf mass) | 1.1 (0.21) | 6.6 (0.62)*** |
| $\delta$-$^{13}$C (‰ vs PDB[b] reference) | −17.18 (1.4) | −29.33 (0.13)** |
| **Soil** | | |
| pH | 4.55 (0.11) | 4.21 (0.09)** |
| Total extractable C ($\mu$g C g soil$^{-1}$) | 27.6 (1.96) | 42.6 (4.84)* |
| Total extractable N ($\mu$g N g soil$^{-1}$) | 12.3 (1.8) | 8.9 (1.6) |
| Total extractable C:N | 3.49 (0.39) | 3.67 (0.33) |
| Microbial biomass C ($\mu$g C g soil$^{-1}$) | 203.2 (29.0) | 147.6 (14.6)** |
| Microbial biomass N ($\mu$g N g soil$^{-1}$) | 41.2 (5.3) | 27.0 (2.6)** |
| Microbial biomass C:N | 4.89 (0.11) | 5.50 (0.19)* |
| TRFLP $H$ | 2.86 (0.14) | 2.63 (0.14)** |
| TRFLP OTU richness ($S$) | 27.6 (3.19) | 21.7 (2.79)* |
| Extractable NH$_4^+$ ($\mu$g N g soil$^{-1}$) | 9.38 (2.1) | 20.25 (3.9)* |
| Extractable NO$_3^-$ ($\mu$g N g soil$^{-1}$) | 16.3 (4.6) | 11.4 (2.2) |
| % C (bulk soil) | 5.13 (0.5) | 5.15 (0.6) |
| % N (bulk soil) | 0.44 (0.05) | 0.40 (0.04) |
| C:N | 11.95 (0.19) | 12.93 (0.21)** |
| $\delta$-$^{13}$C (per mille) (bulk soil) | −20.3 (0.57) | −22.3 (0.35)* |

[a] For all analyses, $n = 8$ paired sites. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values in parentheses are one standard error of the mean.
[b] PDB, Pee Dee belemnite standard.

TABLE 2. Differences between vegetation type for important functional gene groups[a]

| Gene group | Median log fold change | % Difference between vegetation types | No. of probes |
| --- | --- | --- | --- |
| C degradation | −0.021 (0.03)* | −1.45 | 89 |
| Chitinase | −0.11 (0.07)* | −7.53 | 22 |
| Ammonification | −0.16 (0.03)** | −10.7 | 61 |
| N fixation | −0.12 (0.03)*** | −7.85 | 56 |

[a] Values in parentheses are standard errors. *, significantly different from 0 ($P < 0.05$); **, $P < 0.01$; ***, $P < 0.001$.

some kinds of vegetation change. The differences in gene abundance and distribution across sites drove differences in functional gene profiles and led to the plantation sites separating from most of the grassland sites in ordination space (Fig. 1). Both groups that were specific to plantations and those that were common to both vegetation types shared functional genes for chitinases, cellulases, and laccases; however, some functional genes from different strains of the same species could be either restricted to one vegetation type or highly abundant in both, suggesting that functional gene profiles cannot be predicted solely by relative abundances of specific taxonomic groups. The differences between plantations and grasslands seen in the site clusters (see Fig. 3) and in groupings in ordination space likely are driven by groups 1 and 3 (specific to plantations) and by a decreased abundance of functional genes, as discussed below.

In addition to changing the profile of the functional genes present, afforestation led to an overall decrease in abundances of carbon degradation genes (Table 2). The median decrease due to afforestation for carbon degradation probes was 0.021 (log change) or 1.5%, with a range from a 29.3% decrease to an 86.6% increase (Table 2; $P = 0.019$). The most strongly affected subset of carbon degradation probes was chitinases, which decreased on average by 0.113 log (7.5%; $P = 0.017$) (Table 2). The lower relative abundance of these probes contributed to the overall difference seen between grassland and plantations in the functional gene profiles and clusters (Fig. 1).

Changes in carbon degradation genes were accompanied by changes in pool sizes of C in litter and soils. Eucalypt litter had six-times-higher phenolic concentrations and 13% more or-

ganic C than grassland litter had (Table 1). Total extractable C (soluble undigested organic carbon molecules) was 42.6 $\mu$g C g soil$^{-1}$ in eucalypt soil compared to only 27.6 $\mu$g C g soil$^{-1}$ in grasslands ($P < 0.05$) (Table 1); C in microbial biomass under eucalypts was 27% lower than in grasslands ($P < 0.01$) (Table 1). These changes, along with a lower abundance of carbon-degrading functional genes, suggest that the microbial community under eucalypts was less able to degrade the incoming eucalypt litter than grass litter and that carbon in plantation soils is shifting from microbial biomass to total extractable carbon pools, which contain dissolved C that has not been incorporated into biomass (Fig. 1 and Table 1). This conclusion is further supported by the carbon stable isotope results: the $\delta$-$^{13}$C of eucalypt soil is more negative (−22.3) than that of grassland soil (−20.3), which is more similar to the eucalypt litter $\delta$-$^{13}$C than grass litter, suggesting that after only 10 years, much of the eucalypt-derived C has been incorporated and has not been respired (Table 1).

Afforestation of grasslands also altered patterns and abundances of N cycle functional genes. Cluster analysis shows that six out of eight profiles of grassland ammonification genes cluster together (see Fig. 3). In similarity to profiles of carbon degradation genes, many ammonification genes were found in high abundance across both vegetation types (group 1; see Fig. 3), but some were found predominately in plantation soils (see Fig. 3, group 2). The average decrease in abundance for an ammonification gene due to afforestation was 10.7% ($P = 0.0012$) (Table 2). Unlike ammonification genes, N fixation and nitrification genes did not show any distinct clustering patterns due to vegetation type (see Fig. S2 in the supplemental material). However, functional genes for N fixation decreased in average abundance by 7.85% with afforestation as assessed using log change, which evaluates abundance across all related probes ($P < 0.0001$) (Fig. 2 and Table 2). The decreases in relative abundances of functional genes in grassland (both in C and N cycles) and the groups of probes that are specific to plantations caused the differences observed in NMS ordination space between grassland and plantations (Fig. 1).

Changes in N cycle functional genes were accompanied by shifts in soil N pools. Microbial biomass N was 34.5% lower in eucalypt soil than in grassland soil (Table 1). Although the abundance of functional genes for NH$_4^+$ production (ammonification and N fixation) was lower with afforestation, extractable NH$_4^+$ in eucalypt soil was more than twice that in grassland soils (Table 1). Lower microbial biomass N with higher ammonium and lower inputs from ammonification and N fixation could indicate lower microbial assimilation of N after afforestation. Lower assimilation of N by microbes is also sup-
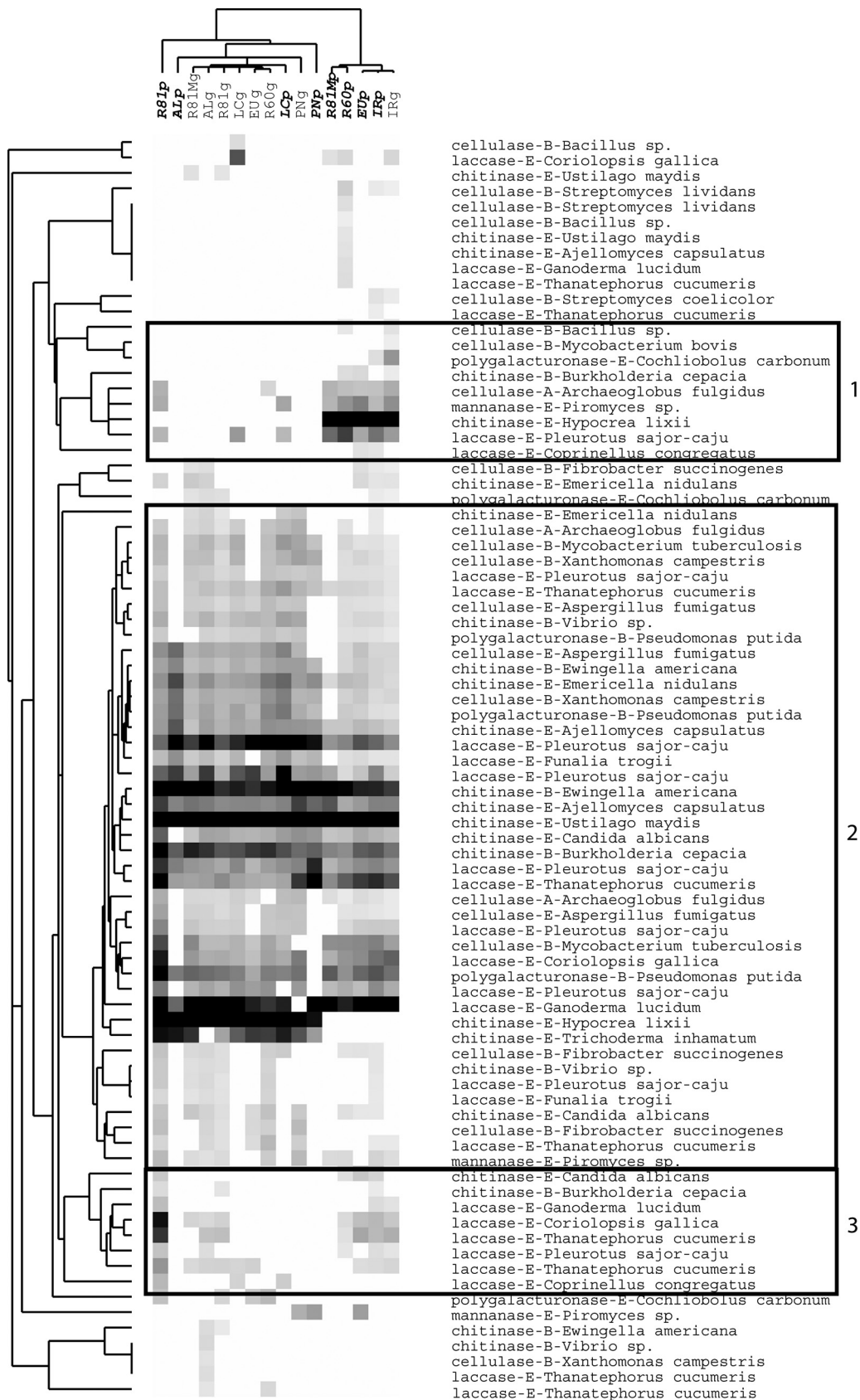
FIG. 2. Cluster analysis of microarray data for probes related to carbon degradation genes. If a probe is white for a given site, then its relative abundance was zero; higher relative abundance is indicated by increased shading. Site names are followed by either a "p" (for plantation, and in italic bold) or a "g" (for grassland). On the *y* axis, probes are identified by their target gene function, followed by domain: A for archaea, E for eukaryotes, and B for bacteria, and the species from which the probe was derived. Boxes identify clusters of functional genes of interest.

ported by the wider C:N ratio of microbial biomass in eucalypt soil and potentially led to the wider C:N ratio of bulk soil under eucalypts (Table 1).

## DISCUSSION

The goal of this study was to assess how different inputs from grasslands and *Eucalyptus* affected soil functions. We found that the vegetation types differed in several ways. *Eucalyptus* litter was higher in phenolic content and percent C than grassland litter was, indicating greater inputs of organic acids to soils. The increased inputs of organic acids, such as phenolic compounds, are a major source of acidity in surface soils (42, 43). In addition, since *Eucalyptus* is evergreen and has no dormant season, its phenolic inputs contribute year-round to soil acidity. The altered litter chemistry inputs from eucalypts versus those from native grasses are a likely reason for the decreased pH we found in surface soils at our sites and the differences in microbial gene structure and abundance (32, 33).

Nonmetric multidimensional scaling of all probes showed that seven of eight grasslands had similar assemblages of functional genes, but the plantation sites separated along both NMS axes showing no consistent grouping (Fig. 1). Site IR's grassland profile was unique in being more similar to several plantation profiles than to any grasslands (Fig. 1). Additionally, site IR's grassland profile of ammonification and carbon degradation functional genes was much more closely clustered with plantations than with other grasslands (Fig. 2 and 3). Site IR was unique in our study in that the grassland was topographically lower than the adjacent plantation and the site had a steep slope from the plantation leading down to the grassland. Through runoff and erosion, this unique topography may have caused the vegetation types to be more hydrologically and biogeochemically connected than the other sites, with the plantation influencing the adjacent grassland profile.

In addition to shifts in overall functional gene structure, afforestation led to decreases in abundance of biogeochemically important genes in several important categories, independently of the taxonomic origin of the probes. Our analysis of specific categories of functional genes suggests that the taxonomic structure of the microbial community has less impact on soil biogeochemistry than the total abundance of functional genes in those categories. This agrees with several recent studies that have shown that microbial functional gene abundance better predicts nitrogen cycling processes than microbial community composition (8, 26, 39). For example, in genes associated with carbon degradation and ammonification, vegetation types clustered in a similar pattern to the NMS (Fig. 1, 2, and 3), but some taxa represented by multiple probes from different sources or strains (e.g., different culture collections or environmental isolates) appeared either as ubiquitous in both vegetation types or specific to only one vegetation type (e.g., *Pleurotus*, *Bacillus*, and *Agrobacterium*).

The decreases in carbon-degrading functional gene potential may have caused the overall decrease in carbon in the microbial biomass with afforestation. In addition, the lower capacity of the microbial community to degrade carbon compounds was correlated with an increased amount of carbon in the total extractable C pool (Fig. 1), and total extractable C was correlated with lower diversity. The total extractable carbon pool represents dissolved carbon substrates that could be respired or mineralized by microbes or eventually incorporated into more-stable soil carbon pools. The accumulation of *Eucalyptus* carbon likely also led to the widening of the bulk soil C-to-N ratio, which indicates less labile material for soil microbial process and could reduce plant productivity in the long term (5, 38). Interestingly, the changes in C degradation and diversity were not correlated with soil pH, which has been shown to be a strong predictor of carbon-degrading genes and diversity across broad scales (18, 19, 48). It has been suggested that pH is an integrator of soil functions, but since the pH variation between vegetation types in this study is relatively small, it might not be an important predictor at this scale.

Changes in vegetation input from grass to *Eucalyptus* also led to a decrease in ammonification and nitrogen fixation genes (Fig. 1). Grassland and *Eucalyptus* sites did not cluster together with probes of nitrogen fixation genes, which also indicates that the changes due to afforestation are not taxon specific (see Fig. S2 in the supplemental material). There are few plants that form symbiotic N-fixing associations in native Pampas grasslands, but rhizosphere-associated N-fixing bacteria are common with grasses; the decrease in N-fixing gene abundance is due either to underrepresentation of N-fixing soil bacteria on this microarray or to losses of these rhizosphere-associated bacteria, which are less common in forests (1, 13, 24). The changes in N fixation and ammonification genes might also be tied to the differences in bacterial diversity; in particular, grasslands tend to have a higher diversity and higher levels of microbial biomass N (Table 1) (see Fig. S1 in the supplemental material).

The lower inputs of mineral N from fixation and ammonification potentially explain the decrease in nitrogen in the soil microbial biomass (Table 1). The changing structure of carbon and nitrogen cycle functional genes also seems to have resulted in a widening of the C:N ratio in microbial biomass. This result could indicate a shift in the stoichiometry of the microbes present due to availability of nitrogen (36). Though the greater abundance of $NH_4^+$ in plantation soils is inconsistent with this conclusion, the overall availability of N (as indicated by the soil C:N ratio and trends toward lower total extractable N and soil N) was lower in plantations (Table 1). In addition, laboratory incubation studies of $NH_4^+$ immobilization in soils of nearby plantations indicated a trend ($P = 0.06$) toward more $NH_4^+$ being immobilized by soil microbes in grasslands (mean, 1.3 μg $NH_4^+$ g soil$^{-1}$ 30 days$^{-1}$) than in plantations (mean, 0.33 μg $NH_4^+$ g soil$^{-1}$ 30 days$^{-1}$) (S. T. Berthrong et al., unpublished data). This suggests that soil microbes in plantations are both less able both to ammonify and fix N and to incorporate it into their biomass. The remaining increase in $NH_4^+$ could be due to the tendency of *Eucalyptus* plantations to take up cations and nutrients from pools deeper in the soil than grasslands, which could lead to lower plant uptake of $NH_4^+$ near the surface of the soils (31, 33).

The presence of a gene does not necessarily mean that it is being actively transcribed, providing some limitations in our ability to draw conclusions about the active function of microbes from these analyses. Analysis of mRNA would allow more direct connections to be drawn. However, recent research on environmental samples using both mRNA and genomic DNA microarrays has shown that the dominant spe-
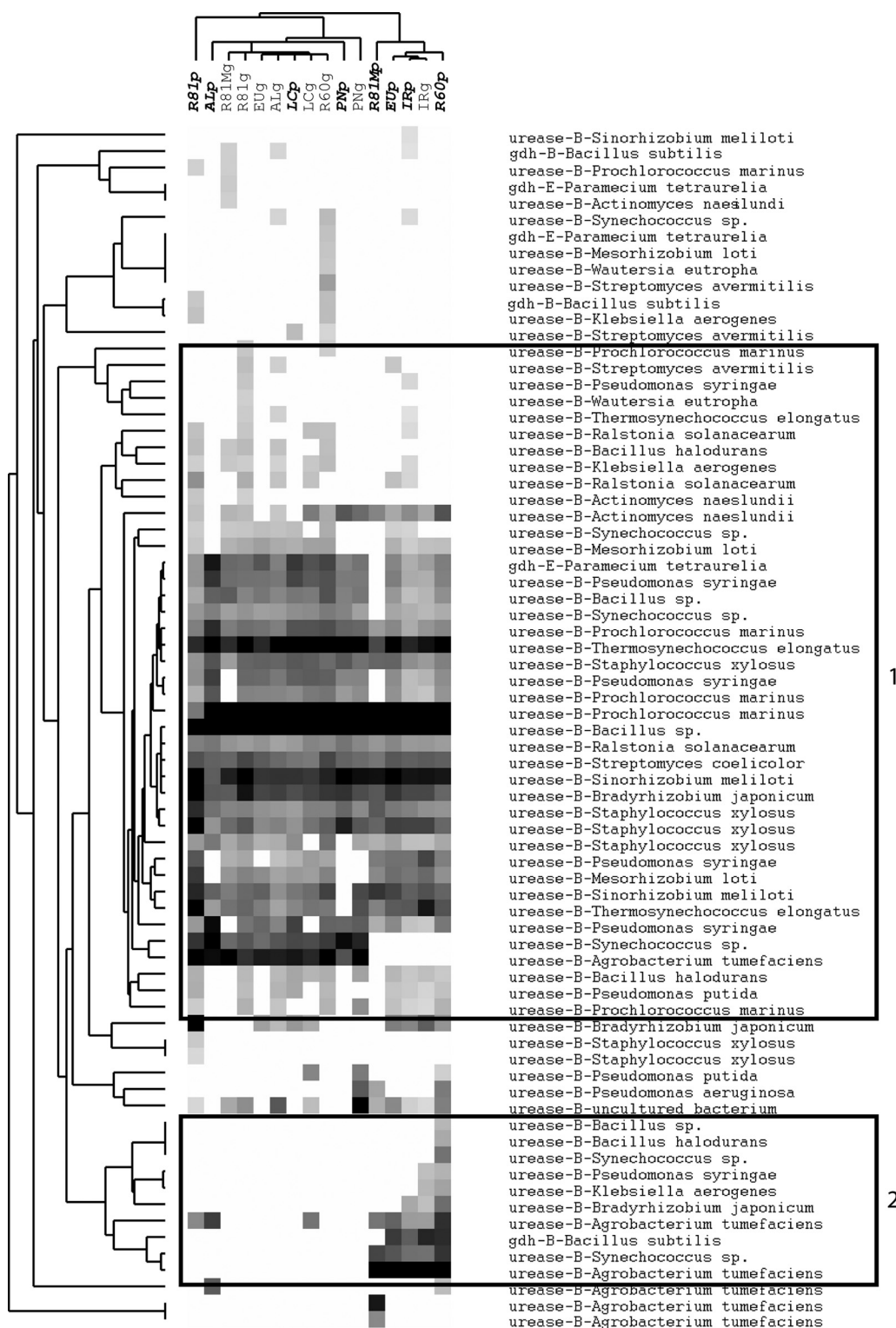
FIG. 3. Cluster analysis of microarray data for probes related to ammonification genes. Please see the legend for Fig. 2 for a description.

cies identified by mRNA arrays are also the most abundant in terms of genomic DNA (6). This suggests that connections drawn between genomic DNA and biogeochemical cycles are reasonable. Nonetheless, future analyses could also incorpo-

rate mRNA transcripts as methods to extract and analyze mRNA from soil continue to improve.

Additionally, the high microbial diversity of soils and the rapid rate of discovery of new knowledge about novel impor-

tant microbial groups and functions make constructing an array that represents all functional groups almost impossible. Our array has several potentially important areas that are underrepresented. For instance, recent research has shown that some archaea can be nitrifiers and are abundant in soil (34). Additionally, it can be difficult to design specific nondegenerate primers for certain soil nitrogen-fixing bacterial genes (7, 44). Despite these limitations, our results indicate links between microbial functional genes and biogeochemical pools, suggesting that these microarrays cover a large and important part of the soil microbial population in these soils.

Microarrays are emerging as a valuable tool for evaluating ecological questions by analyzing biogeochemically relevant genes in a parallel manner. In agreement with our hypotheses, this study shows that afforestation of grasslands led to a decrease in abundance of several important gene families important for soil C and N cycling. The functional gene differences and shifts in biogeochemical cycles between grasslands and plantations suggest there are detectable links between microbial community functional gene composition and biogeochemical functions. The differing microbial functions between grasslands and plantations were also accompanied by a more acidic soil and altered soil C and N pools (e.g., soil $^{13}$C and soil C:N ratio). In the long term, these biogeochemical shifts might have important implications for both the productivity of successive rotations of plantations and the rate at which carbon and nitrogen are incorporated into soil.

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