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REGULAR ARTICLE

Pine afforestation alters rhizosphere effects and soil nutrient turnover across a precipitation gradient in Patagonia, Argentina

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Abstract
Aims Pine species, when planted outside their native range, can have profound impacts on soil carbon (C) and nitrogen (N) pools, which may be related in part to their association with ectomycorrhizal fungi. We investigated the effects of exotic planting of *Pinus ponderosa* on C and N cycling in the rhizosphere along a precipitation gradient in Patagonia, Argentina.
Methods We measured C mineralization, microbial biomass-C, and soil enzyme activity in rhizosphere and bulk soil collected from pine plantations and native vegetation. Rhizosphere effects were calculated as the percent difference between paired rhizosphere and bulk soil samples.
Results In afforested sites, we found enhanced rhizosphere effects for C mineralization and microbial biomass relative to native vegetation, but not for enzymatic activity. The absolute values of all evaluated variables were significantly reduced in pine plantations compared to native vegetation. We also observed strong correlations between enzymatic activity, and soil organic matter and microbial biomass. For both pine and native trees species, rhizosphere effects for N-degrading enzymes were positively correlated with precipitation.
Conclusions Pine afforestation reduced overall N turnover and microbial activity in these ecosystems. Our data suggest that these reductions may be driven by reductions in soil organic C pools. Additionally, ecosystem water availability may directly or indirectly regulate the magnitude of rhizosphere effects on N cycling independent of plant species. The negative impacts of afforestation on N cycling should be considered in evaluating the long-term potential for C sequestration in these human modified ecosystems.

Keywords Afforestation · Patagonia · Pine · Rhizosphere effects · Soil enzyme activity · *Nothofagus* spp. · Nitrogen · Soil organic matter

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Introduction

The introduction of species outside their native ranges, whether intentional or unintentional, can have profound

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impacts on ecosystem-level biogeochemical cycling (Liao et al. 2008; Berthrong et al. 2009). While plants may influence nutrient cycling through a suite of traits, increased attention has been paid recently to linkages with belowground biota (Eviner and Chapin 2003; Wardle et al. 2004; Chapman et al. 2006). For example, mycorrhizal fungi, which form symbiotic associations with plant roots, play an important role in plant nutrient uptake (Read and Perez-Moreno 2003). Ectomycorrhizal (ECM) fungi have been shown to be capable of mobilizing nitrogen (N) from detritus, and of producing enzymes that decompose large organic compounds like lignin and cellulose, in addition to enzymes which hydrolyze N-containing compounds like proteins and chitin (Read and Perez-Moreno 2003; Courty et al. 2005; Courty et al. 2006; Talbot et al. 2008). Thus, ECM fungi may not only facilitate greater access by plants to N physically, because their hyphae are able to explore more soil volume than roots, but also biochemically, through decomposition and access to N stored in recalcitrant organic material.

Introduced tree species, and particularly pine species, are often utilized in plantation forestry, preferred for their fast growth and elevated production of plant biomass relative to native species (FAO 2006; Lal 2008). Planted forests represent an important land use change globally; as of 2010, they accounted for 264 million hectares worldwide, and the area of land dedicated to this activity is growing steadily (FAO 2010). Afforestation with pine species generally results in a reduction of soil carbon (C) and N pools (Paul et al. 2002; Berthrong et al. 2009; Hess and Austin 2014). Increased productivity of pine plantations compared to the native vegetation they replace may be associated with elevated plant N demand (Luo et al. 2004; Norby et al. 2010; Gelfand et al. 2012), and diminished soil N stocks following afforestation with pine species supports the idea of a transfer of N from soil to tree biomass (Richter et al. 2000; Kirschbaum et al. 2008; Berthrong et al. 2009; Hess and Austin 2014). Replacement of native vegetation with pines may also result in increased ecosystem-level N use efficiency (Gelfand et al. 2012) and improved synchronization between inorganic N availability and plant demand (Gelfand and Yakir 2008). While many studies have focused on the impacts of afforestation on C cycling (e.g. Bárcena et al. 2014), less is known about how afforestation affects N cycling, and how alterations to N cycling may affect long-term C storage in plantations (Hess and Austin 2014).

Plants can dramatically modify the soil environment surrounding their roots—the rhizosphere—with fluxes of C stimulating increased microbial activity, decomposition, and availability of nutrients for plant uptake (Fontaine et al. 2003). Stimulation of soil organic matter decomposition by C flow into the rooting zone has been called the priming effect (Fontaine et al. 2003). Because decomposition of C and N tend to be linked, the stimulation of microbial activity and decomposition in the rhizosphere could also stimulate nutrient cycling and release (Jones et al. 2004; Dijkstra et al. 2009). Furthermore, high microbial activity in the rhizosphere has been linked to accelerated turnover of soil organic matter (Phillips et al. 2012) and increased N availability (Brzostek et al. 2013) in temperate forests.

"Rhizosphere effects" have been measured empirically in a number of ways. They have been inferred from the difference in variables of interest (e.g. C mineralization) between potted soils grown with and without plants (e.g. Bader and Cheng 2007). Soils have been sampled with varying proximity to roots in mesocosms (e.g. Norton and Firestone 1991). Under field conditions, rhizosphere soil may be collected by gently scraping soil attached to excavated roots. Rhizosphere effects may then be inferred from absolute differences in variables of interest in rhizosphere soil compared to bulk soil (soil not adhering to roots) (e.g. Hernesmaa et al. 2005), or as the percent difference between rhizosphere and bulk soil (Phillips and Fahey 2006). The method used to measure and calculate rhizosphere effects can influence the magnitude and/or significance of the reported results, and thus should be taken into account when interpreting results across studies.

Recent work has suggested that mycorrhizal type and plant identity may influence the magnitude of rhizosphere effects for soil microbial activity, with ectomycorrhizal tree species having greater rhizosphere effects (defined as the percent difference between rhizosphere and bulk soil) than those forming associations with arbuscular mycorrhizal fungi (Phillips and Fahey 2006). However, little is known about how rhizosphere dynamics vary among different plant species and their associated mycorrhizal symbionts, particularly in a field context (Dijkstra et al. 2009). Most studies of rhizosphere effects have been conducted with herbaceous species, and less is known about rhizosphere effects in tree species (Bader and Cheng 2007). Results of several pot experiments suggest that pines may have higher microbial activity in the rhizosphere compared to other

species, such as *Quercus* spp. (Trocha et al. 2010). In 18-year-old plantations, Zhao et al. (2010) found higher rhizosphere effects for *Pinus sylvestris* than for *Populus simonii* and *Ulmus pumila* for net N mineralization and nitrification. Using ^{14}C signatures in fruiting bodies, Chapela et al. (2001) showed that pine ECM were capable of metabolizing soil C, and linked this to large losses in soil C following pine afforestation of grasslands. Nuñez et al. (2009) also found that the presence of ECM played an important role in the success of invasions of pines outside their native range. Together, these studies suggest the importance of ECM associations in pines for tree growth as well as soil nutrient cycling. Finally, little is known about how water availability affects the magnitude of rhizosphere effects, although again, pot experiments with herbaceous species suggest that priming effects may be greater at high soil moisture (85% of field capacity) compared to low soil moisture (45% of field capacity; Dijkstra and Cheng 2007).

In most ecosystems and soils, the bulk of soil N is in organic forms inaccessible to plants. As such, microbial production of extracellular enzymes that depolymerize macromolecules to soluble monomers in the rhizosphere may mediate plant N availability (Hawkes et al. 2007). These monomers are then available both to plants and microbes for uptake, or to microbes for mineralization. From the perspective of the microorganisms that produce them, the primary purpose of enzyme production is resource acquisition (Allison et al. 2010). At the ecosystem level, extracellular enzymes mediate rate-limiting steps in decomposition and mineralization. In spite of the importance of extracellular enzymes in soil function, relatively little is known about the factors that regulate their production. Enzyme production may be inhibited by the presence of products and/or assimilable labile resources; it may also be stimulated by the presence of substrate (Allison and Vitousek 2005; Allison et al. 2010). Thus, enzyme activity reflects microbial nutrient demand, determined both by environmental nutrient availability as well as the stoichiometry of the microbial biomass (Allison et al. 2010). On a global scale, hydrolytic soil enzyme activities have been correlated with pH and soil organic matter, and less strongly with climatic variables, i.e. mean annual temperature and precipitation (Sinsabaugh et al. 2008).

In this study, we explored the effects of exotic pine species and climate on plant-microbe-soil interactions in northwest Patagonia, Argentina. In this region, forest plantations were heavily subsidized in the mid 1970s through credit and tax exemptions, and are predominantly composed of *Pinus ponderosa* (Laclau and Pozo 2011). Estimates from over a decade ago put the area afforested with *P. ponderosa* in the region at over 70,000 ha, growing at a rate of 5000 ha per year (Laclau 2003). An abrupt rainfall gradient in a relatively small geographic region also means that plantations are found in areas with a wide range of mean annual precipitation (MAP), affording the opportunity to study interactions between the impacts of afforestation and those of climate on biogeochemical cycling. We selected five paired sites, each with native vegetation and an adjacent pine plantation, along a gradient from 250 to 2200 mm MAP. We hypothesized that rhizosphere effects for soil microbial activity would be greater in introduced pines relative to native ectomycorrhizal trees, which could indicate greater access to and acquisition of N by pines, a potential mechanism explaining elevated primary production as well as reduced soil N in pine plantations relative to native vegetation in these sites.

Materials and methods

Study site

Our research took advantage of an unplanned natural experiment in northwest Patagonia, Argentina, where areas of steppe and native forest have been afforested with a single exotic species of pine, *P. ponderosa*. We identified a set of five paired sites, each with native vegetation and an adjacent pine plantation, along a 61 km east-west precipitation gradient from 250 to 2200 mm MAP at 40° S ± 0.5° in Neuquén, Argentina (for a map of study location: Supporting Information Fig. S1, Hess and Austin 2014). The sites cover an area ranging from Junin de los Andes, the watersheds of rivers Quemquemtreu and Curruhue, and lakes Meliquina, Lolog, and Lácar. Precipitation is concentrated between May and September, with dry summers (Paruelo et al. 1998). The abrupt changes in precipitation in this region are due to the interaction between the Andes mountains and the westerly winds from the Pacific ocean, which cause an exponential decline in precipitation over a distance of less than 100 km (Jobbágy et al. 1995; Austin and Sala 2002). Average temperature varies between 8.9° and 12 °C independently of changes in MAP, with minimum temperature below 0 °C in June and July. The dominant life forms and

species composition of the native vegetation in the five sites along the precipitation gradient are as follows: 1 and 2) semi-arid shrub-grass steppe (250 mm and 650 mm MAP); 3 and 4) mixed southern beech (*Nothofagus antarctica*) woodland (1100 mm and 1350 mm MAP); and 5) mixed southern beech (*N. dombeyi, N. nervosa, and N. obliqua*) closed-canopy forest (2200 mm MAP) (Table 1). *Nothofagus* spp.—the dominant vegetation in the wettest three sites—is a dominant overstory species of humid Andean Patagonian forests (Veblen et al. 1996). Furthermore, to our knowledge, *Nothofagus* spp. is the only genus of native trees in Patagonia that forms ECM associations (Andrade et al. 2000; Fontenla et al. 2001; Alvarez et al. 2009).

Little information exists regarding soils in the Andean Patagonia region of Argentina. Soils in this region were formed from tephra from various volcanic eruptions, the most recent from 1960 and the oldest from approximately 10,000 years ago (Broquen et al. 2005). Upper soil horizons were likely all formed in the same tephra (Broquen et al. 2005), although the drier sites (sites 1 & 2) have additionally been influenced by sand and fluvial clay deposition (Etchevehere and Dimitri 1972). Government maps indicate soils in sites 3–5 are Entisols (Table 1), while other studies have classified them as Andisols (Broquen et al. 2005). These soils have high volcanic glass content (Broquen et al. 2005). Although soils in all sites are young and have experienced low weathering, previous work has found that weathering increases with MAP in this region, along with the content of amorphous aluminum and iron minerals (i.e. allophane and imogolite; Broquen et al. 2005). Soil texture also likely transitions from sand and sandy loam in sites 1 & 2 to silt in the wetter sites (Broquen et al. 2005). Soil pH across sites varies between 6.1 and 6.8, with no significant differences between pine plantations and sites with native vegetation (Araujo 2012).

In each site, we established a representative parcel of 50 m × 50 m (10 parcels in total), in which all sampling was conducted. The slope (<5%) and orientation of the plots were similar in all cases, and with the exception of the most humid site, the pine plantation and native vegetation were part of a continuous area, with the paired plots adjacent to one another. All plantations were planted with *P. ponderosa* between 1974 and 1976, such that all plantations sites were 34–36 years old, with the same initial planting density. The establishment of the plantations did not involve removal of the understory vegetation or application of synthetic chemicals. In the wettest site, native forest vegetation had been selectively cut for two decades before the seeding of the pine plantations (E. Coliqueo, personal communication), and in the savannah sites (sites 3 & 4), native *Nothofagus* spp. were selectively removed for plantation establishment. Plantations have not been irrigated or fertilized since they were planted, and were not pruned or thinned during the period of the study.

Sample collection

Data from two field seasons are presented in this study. Soils were collected in summer (December) 2009 and again in summer (December) 2010. Soils in summer 2009 were collected along transects and were used for measurement of enzyme activity and organic matter determination. Soils to perform rhizosphere experiments were collected in summer 2010 and were used to evaluate enzyme activity, microbial biomass-C, and C mineralization rates.

Table 1 Site location and characteristics. Mean annual precipitation (MAP) is based on long-term averages (Araujo 2012; Araujo and Austin 2015)

Site	(mm)	Location	MAT (°C) (min – max)	Altitude (m)	Soil type (GeoInta 2012)	Dominant native vegetation type
1	250	40° 15′ S 70° 48 W	12 (−0.5 – 29)	851	Natrixeralf	Shrub-grass steppe
2	650	39° 57′ S 71° 06′ W	9.5 (−1.4–4.9)	1024	Argixeroll	Shrub-grass steppe
3	1100	40° 26′ S 71° 13′ W	8.9 (−0.9–2.6)	906	Dystrandept	*Nothofagus antarctica* woodland
4	1350	40° 04′ S 71° 20′ W	11 (2–21.4)	1062	Vitrandept	*N. antarctica* woodland
5	2200	40°09'S 71°34'W	9,7 (−0.1–4.4)	804	Dystrandept	*N. dombeyi*, *N. nervosa*, & *N. obliqua* forest. *Chusquea culeou* understory.

In summer 2009, eight soil cores were collected to a depth of 10 cm in each site, at random along 4 transects lain at 10 m intervals (2 samples per transect). Soil was stored in ziplock bags at 4 °C until processed. Soils in this region have A horizons to a depth of 13–43 cm (Broquen et al. 2005), and thus our sampling to 10 cm should have only collected soils from only A horizons consistently in all sites.

In summer 2010, soil sampling and processing was conducted based on a modified version of sample collection described in Phillips and Fahey (2006). In all sites except those with native vegetation in sites 1 and 2, five representative trees were selected for sampling. The native ecosystems in sites 1 and 2 are shrub-grass steppe and do not contain vegetation that form associations with ECM, and thus were not sampled. In pine plantations, *P. ponderosa* was sampled; in sites with native vegetation, the dominant species, *Nothofagus* spp., were sampled. In sites 3 and 4, *N. antarctica* was sampled, and in site 5, *N. dombeyi*, *N. nervosa*, and *N. obliqua* were sampled (n = 5 for each species). Soil blocks 10 cm × 10 cm × 10 cm were removed from 3 points around each tree, spaced as equidistantly from each other as possible to capture spatial variability in the root system, approximately 1 m from the trunk. These samples were then pooled to make one composite sample per tree. The proximity to the trunk ensured that the majority of roots sampled were from the tree in question; however, the inclusion of roots from neighboring trees would not invalidate our analysis, as our interest was in measuring rhizosphere effects representative of the site as a whole. In addition, four soil samples were collected in each site from areas as far as possible from neighboring trees, for general site characterization purposes. Soil samples were stored in ziplock bags at 4 °C until processed.

Soil processing

All processing and analysis was performed at the College of Agronomy, University of Buenos Aires. For soils for rhizosphere experiments, rhizosphere soil was separated from bulk soil shortly after field collection. Fine roots and adhering rhizosphere soil were carefully picked out of soils with forceps and shaken gently to remove loose soil. The characteristic structures of ectomycorrhizal symbioses (mantle, rhizomorphs, and Harting net) were present in all individuals of the four species of *Nothofagus* in the sites where they were present, as well as in pines in all sites. Soil adhering to fine roots after gentle shaking was defined as rhizosphere soil, while that not adhering was defined as bulk soil. Although it is possible that ECM hyphae were present in bulk soil, this collection method allowed for comparison between rhizosphere and bulk soil in close spatial proximity, thus reducing noise from soil heterogeneity across larger spatial scales. Rhizosphere soil was carefully removed from fine roots by gentle scraping with fine forceps and small brushes. Because of the time-intensiveness of this process, 20-25 g rhizosphere soil was collected per sample.

Roots were removed and soils were sieved prior to all analysis. Subsamples of all soils were dried at 100 °C to determine water content, to convert wet weight to dry weight.

Laboratory analysis

Soil enzyme activity

The potential activity of four soil enzymes was measured in all soils collected: beta-glucosidase (BG), glycine aminopeptidase (GAP), N-acetyl-glucosaminidase (NAG), and polyphenol oxidase (POX) (Table S1). For the hydrolytic enzymes (BG, GAP, and NAG), hydrolysis of substrate side groups linked to p-nitrophenyl (pNP), a chromogenic moiety, produces p-nitrophenol, which has an intense yellow color. Measurement of POX activity is based on the oxidation of a phenolic amino acid. Enzyme substrates were purchased from Sigma Aldrich (St. Louis, MO).

Enzyme activity was measured using techniques modified from Sinsabaugh et al. (1999) and Allison and Jastrow (2006). Soils were homogenized and mixed with distilled water in a ratio of 1 g soil: 10 mL water. Substrates were dissolved in tris buffer (pH 7.2) for assays with soils from sites 1, 2, and 3, and acetate buffer (pH 5.5) for assays with soils from sites 4 and 5, to most closely match original soil pH. In centrifuge tubes, 1 mL homogenate was combined with 1 mL substrate-buffer solution and agitated at 25 °C for 2 h. Sample controls (buffer + homogenate) and substrate controls (buffer + substrate) were included. After incubation, tubes were centrifuged for 10 min and 1 mL supernatant was extracted. Absorbance was read at 410 nm for BG, GAP, and NAG samples in a Spectrum SP-1105 Visible Spectrophotometer. To BG and NAG samples, 0.1 mL 1 M NaOH was added before

measurement in the spectrophotometer. Absorbance was read at 460 nm for POX samples.

Enzyme activity is expressed as μmol substrate converted per hour per gram dry mass as follows (Sinsabaugh et al. 1999):

$$\text{Activity}\left(\mu\text{mol h}^{-1}\text{g}^{-1}\text{DM}\right) = \text{OD}/(\text{EC x T}_h\text{x DM})$$

where

OD	sample absorbance – (substrate control absorbance + sample control absorbance)
EC	micromolar extinction coefficient (EC/μmol) for p-nitrophenol and DOPA, approximately 1.6
T_h	incubation period in hours
DM	dry mass of soil, as g dry soil/mL sample homogenate

Soil microbial biomass-C and C mineralization rates

Microbial biomass-C (MB-C) and C mineralization rates were quantified for soils collected in summer 2010 using a modification of the chloroform fumigation-incubation method described in Paul et al. (1999) and Phillips and Fahey (2006). Soils were sieved, and 5 g of field moist soil was fumigated with chloroform for 18–24 h in a humid environment. Fumigated soils were then re-inoculated with 0.25 g unfumigated soil and incubated in a 380 mL glass jar for 10 days. Unfumigated controls were also incubated for 10 days in the same jars under the same conditions, serving both as controls and to quantify potential soil C mineralization rates. Base traps with 4 mL 0.1 M NaOH (for sites 1, 2, and 3) and 0.2 M NaOH (for sites 4 and 5) were used to collect CO_2 released from incubating soil. Blanks were included. Double end-point titration (to pH 8.3 and pH 3.8, respectively) was used to determine CO_2 released using a Mettler Toledo DL-15 titrator (Mettler Toledo, Greifensee, Switzerland).

Soil organic matter

Percent soil organic matter was determined for all soil samples from summer 2009 through the ignition loss method. For all samples, a known quantity of dry soil (dried 48 h at 100 °C) was ground and placed in a muffle furnace and heated to 450 °C for 4 h. After cooling to room temperature, the sample was weighed again to determine mass loss of soil organic matter.

Statistical analysis

All statistical analysis was performed in R 2.15.1 (R Core Team 2012). Average values for each response variable in each microsite (rhizosphere, bulk, general site) were calculated by site and species. One-way ANOVA was performed to identify differences in means of response variables between the three *Nothofagus* spp. in site 5; once no significant differences were identified, response variables were averaged and one value reported for *Nothofagus* spp. in site 5. Three-way ANOVA was performed on soil enzyme activity, MB-C, and C mineralization rates from summer 2010, with MAP, species (*Nothofagus* spp. vs. pines), and microsite (rhizosphere vs. bulk) as factors. General site-level soil samples were not included, since comparing rhizosphere and bulk samples was prioritized. Only sites 3–5 were used in the analysis, since sites 1 and 2 only included data from pines. Log and/or box-cox transformations were performed on data that did not meet conditions of normality and homoscedasticity per the Shapiro Wilk Normality Test and Levene's Test, respectively. For all tests, $\alpha = 0.05$.

We adopted the definition of the "rhizosphere effect" from Phillips and Fahey (2006). The rhizosphere effect was calculated as the percent difference between paired rhizosphere and bulk soil samples for a given soil variable, that is, the difference between rhizosphere and bulk values, divided by the bulk value. A positive rhizosphere effect indicates a greater pool or rate in the rhizosphere soil relative to bulk soil, while a negative effect indicates a greater pool or flux in the bulk soil. 95% confidence intervals were calculated for rhizosphere effects for all response variables, by species (pines or *Nothofagus* spp.) and MAP. If the confidence interval included zero, the rhizosphere effect was considered to be not statistically different from zero. Two-way ANOVA (with MAP and species as factors) was also performed with rhizosphere effects.

A Model II standard major axis regression— to account for error in variables on both axes (Legendre 1998)—was conducted to relate microbial acquisition effort for C to microbial acquisition effort for N (Sinsabaugh et al. 2009). The natural log of BG activity was regressed against the natural log of combined GAP and NAG activity, using all sites and microsites (where appropriate) in each data set, for soil samples taken in both field seasons. Because lignin is not a primary C source for any major group of microorganisms, POX

activity was not included in this ratio (Sinsabaugh et al. 2008). Regressions were also calculated for each microsite (when appropriate) and by vegetation type for both field seasons, to identify any differences in slope. The same regression model was also used to relate enzyme activity to organic matter concentration for soils collected in summer 2009, and soil enzyme activity to MB-C for soils collected in summer 2010.

Results

For all variables, MAP, species identity, and microsite (rhizosphere vs. bulk) were significant predictors of microbial activity (Table 2; Figs. 1, 2). All variables were higher in rhizosphere soils compared to bulk soils, and in soils sampled under *Nothofagus* spp. compared to those sampled under pines. MAP and species also demonstrated significant interactions, with a generally greater positive effect of precipitation on microbial activity in soils from *Nothofagus* spp. and a lesser positive or no effect of precipitation on microbial activity in soils from pines. For POX activity, however, MAP and species interacted such that precipitation had a *negative* effect in pines and no effect in *Nothofagus* spp. (Table 2, Fig. 1).

Rhizosphere effects as we defined them—the percent difference between paired rhizosphere and bulk soil samples—for enzyme activities were variable, with no consistent significant rhizosphere effects by species, site, or enzyme (Table 3). One exception to this was in *Nothofagus* spp. in site 5 (the 2200 mm site), which showed positive rhizosphere effects for all enzymes. At the same time, rhizosphere effects were positively correlated with site MAP for GAP and NAG activity, and were higher in *Nothofagus* spp. compared to pines for POX activity only. Rhizosphere effects for soil MB-C and C mineralization rates were similarly variable to those for enzyme activities (Table 4); however, rhizosphere effects were consistently larger in pines than in *Nothofagus* spp. (Table 2).

Enzymatic activity related to C acquisition was correlated to enzymatic activity related to N acquisition; the natural log of the combined activity of GAP and NAG showed a strong linear relationship to the natural log of BG activity (Fig. 3) in soils from both field seasons. Slopes of regressions by microsite, vegetation type, and field season were not significantly different from each other (Table 5). Hydrolytic soil enzyme activities were linearly related to soil organic matter content, although POX showed no relationship to SOM (Fig. 4). All enzyme activities also showed linear relationships to soil MB-C (Fig. 5).

Table 2 ANOVA results

Variable	Factor			
	MAP	species	microsite	MAP x species
BG activity, summer 2010	101.7***	184.1***	7.3**	9.0**
GAP activity, summer 2010	201.4***	373.8***	8.7**	7.7**
NAG activity, summer 2010	118.0***	40.7***	4.2*	31.3***
POX activity, summer 2010	n.s.	134.3***	7.0*	15.5***
MB-C, summer 2010	94.4***	162.0***	11.3**	19.7***
C mineralization, summer 2010	84.0***	127.8***	35.8***	11.2**
Rhizosphere effects, BG activity	n.s.	n.s.	n.s.	-
Rhizosphere effects, GAP activity	7.1*	n.s.	n.s.	-
Rhizosphere effects, NAG activity	8.3**	n.s.	n.s.	-
Rhizosphere effects, POX activity	n.s.	15.6***	n.s.	-
Rhizosphere effects, MB-C	n.s.	15.9***	n.s.	-
Rhizosphere effects, C mineralization	n.s.	10.0**	n.s.	-

F values are reported with p values indicated as follows: *** for $p < 0.001$, ** for $p < 0.01$, and * for $p < 0.05$. MAP x microsite, species x microsite, and MAP x species x microsite interactions were not significant for any variable. Rhizosphere effects were calculated as the percent difference between paired rhizosphere and bulk soil samples for a given soil variable

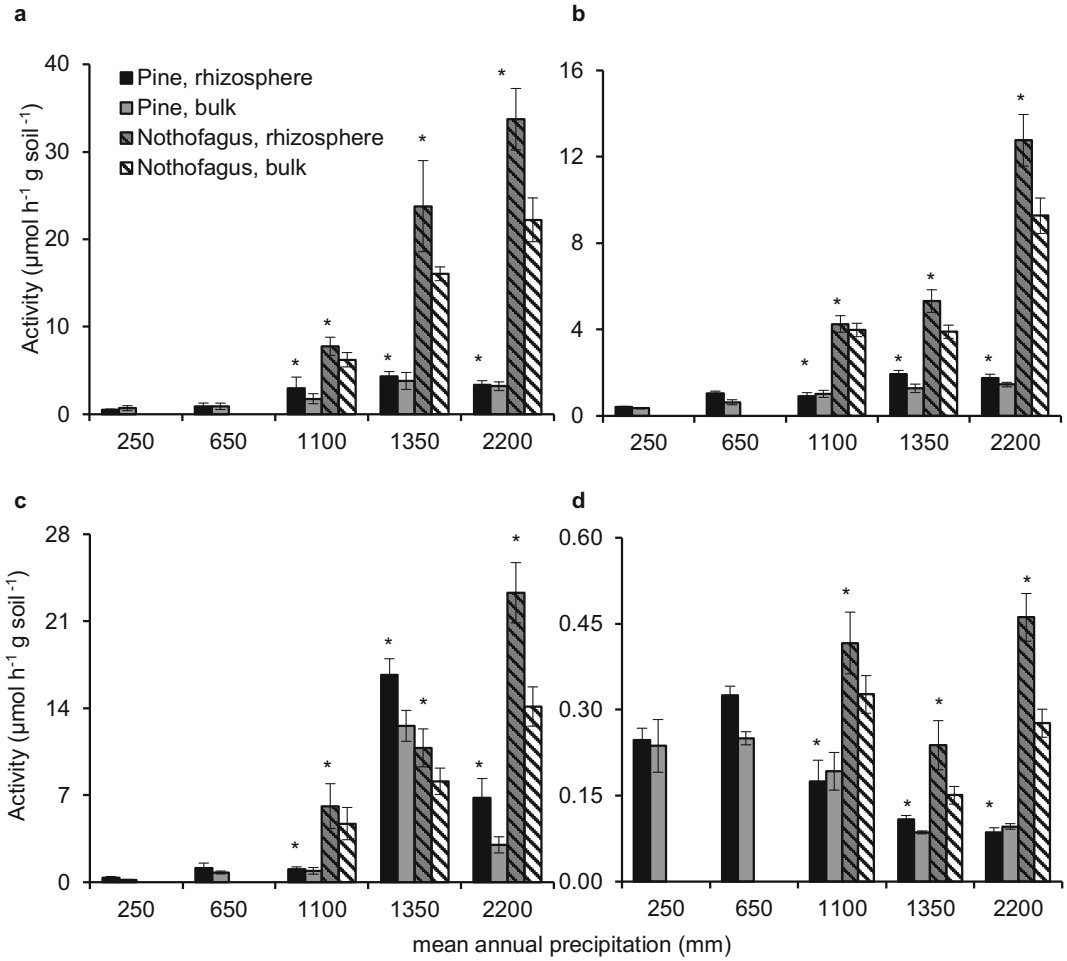

Fig. 1 Enzyme activity in rhizosphere and bulk soil along the precipitation gradient, in pine plantations and sites with natural vegetation. **a** BG activity. **b** GAP activity. **c** NAG activity. **d** POX activity. Error bars represent standard errors. Asterisks over rhizosphere soils indicate that enzyme activity in rhizosphere soils was significantly greater than that in bulk soil, as indicated by ANOVA

Discussion

Rhizosphere effects in native and afforested ecosystems in Patagonia

The effect of roots on microbial activity may have important consequences for soil organic C storage and nutrient availability at the ecosystem scale (Phillips et al. 2012; Brzostek et al. 2013). In spite of their potential importance, few studies to date have evaluated rhizosphere effects for mature trees in forest ecosystems in a field context. In this study, we found enhanced microbial activity in rhizosphere soils compared to bulk soils under both pines and *Nothofagus* spp. in all the sites across a range of MAP (Figs. 1 and 2; Table 2). These results are consistent with results from a previous study in these sites indicating that ectomycorrhizae figure prominently in the N nutrition of these tree species (Hess and Austin 2014). However, we found mixed support for our hypothesis that pines would have greater rhizosphere effects for microbial activity than *Nothofagus* spp., evaluated across a wide range of precipitation. While pines appeared to have greater rhizosphere effects relative to native trees for microbial biomass and C mineralization rates, these effects did not extend to potential extracellular enzyme activity. As such, enhanced rhizosphere effects on soil enzymatic activity per se do not appear to be a mechanism that contributes to the persistent success of this pine species and its higher productivity

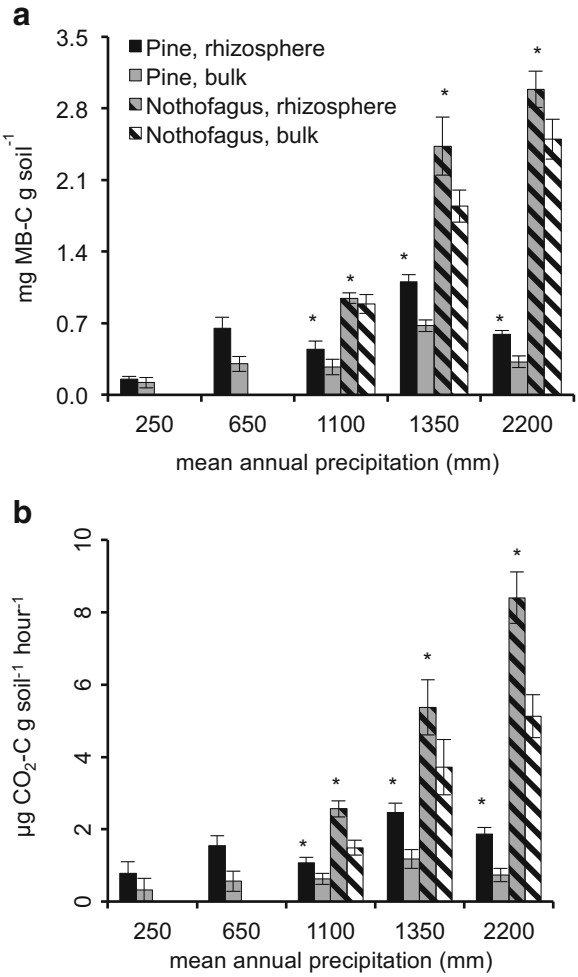

Fig. 2 Soil MB-C (**a**) and C mineralization rates (**b**) in pine plantations and sites with native vegetation along the precipitation gradient. Error bars represent standard errors. Asterisks over rhizosphere soils indicate higher values in rhizosphere soils compared to bulk soils, as indicated by ANOVA

Table 4 Rhizosphere effects for soil microbial biomass C and C mineralization rates for all levels of precipitation, in *P. ponderosa* and *Nothofagus* spp. Values represent the percent difference between rhizosphere and bulk soil samples with 95% confidence intervals

Species	MAP	Microbial biomass C	C mineralization
Pinus ponderosa	250	191 ± 328	197 ± 247
	650	191 ± 327	**192 ± 162**
	1100	141 ± 222	96 ± 104
	1350	**67 ± 39**	**110 ± 34**
	2200	119 ± 159	**191 ± 147**
Nothofagus spp.	1100	12 ± 55	**72 ± 32**
	1350	28 ± 54	58 ± 67
	2200	**20 ± 18**	**66 ± 31**

Numbers in bold indicate that the confidence interval does not contain zero and the effect is statistically significant

compared to native tree species in the region (Laclau 2003; Araujo 2012).

There are many reasons why enhanced rhizosphere effects for microbial biomass and C mineralization in pines compared to *Nothofagus* spp. may not have translated into effects on potential soil enzyme activity. Soluble, low molecular weight rhizodeposits (Cheng and Gershenson 2007) could have been assimilated directly by microbes and respired, obviating the need for the action and thus production of extracellular enzymes. Given more labile C availability, microbial investment may have been in growth and reproduction (evidenced by increased C mineralization and microbial biomass), but without new investment in enzyme synthesis (Schimel and Weintraub 2003). Also, we measured enzyme activity potentials, not in situ activities,

Table 3 Rhizosphere effects for soil enzyme activities for all levels of precipitation, in *Pinus ponderosa* and *Nothofagus* spp. Values represent the percent difference between paired rhizosphere and bulk soil samples for each enzyme activity with 95% confidence intervals

Species	MAP	BG	GAP	NAG	POX
Pinus ponderosa	250	-2 ± 49	19 ± 40	123 ± 128	17 ± 52
	650	-2 ± 81	92 ± 113	43 ± 95	**30 ± 18**
	1100	59 ± 67	**-13 ± 11**	47 ± 99	-8 ± 31
	1350	36 ± 83	**61 ± 49**	39 ± 61	**26 ± 21**
	2200	14 ± 58	21 ± 24	**128 ± 80**	-10 ± 24
Nothofagus spp.	1100	28 ± 34	10 ± 41	**28 ± 15**	**27 ± 22**
	1350	45 ± 71	**36 ± 19**	38 ± 66	73 ± 144
	2200	**72 ± 62**	**50 ± 30**	**80 ± 37**	**77 ± 28**

Numbers in bold indicate that the confidence interval does not contain zero and the effect is statistically significant

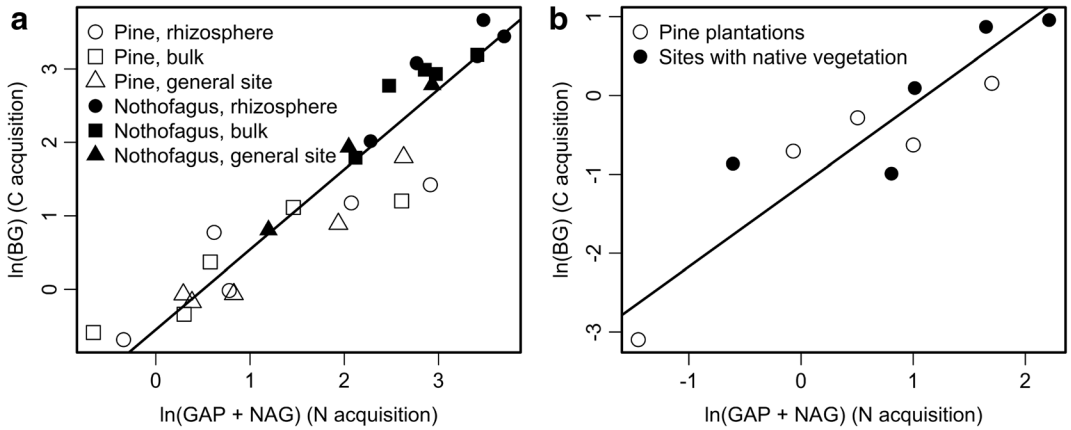

Fig. 3 Organic nitrogen acquisition activity in relation to carbon acquisition activity across sites and microsites. BG activity is used as an indicator of potential carbon acquisition, and the combined activities of GAP and NAG are used as an indicator of potential nitrogen acquisition. **a** summer 2010. $r^2 = 0.88$, $p < 0.001$. $y = 1.090x - 0.549$. **b** summer 2009. $r^2 = 0.80$, $p < 0.001$. $y = 1.027x - 1.144$

which may be affected by endogenous soil moisture and substrate availability, and adsorption to soil particles, among other factors (Wallenstein and Weintraub 2008). Lab-based assays of enzyme activity are conducted with non-limiting amounts of substrate, and include the activity of enzymes stabilized or adsorbed to soil particles (Wallenstein and Weintraub 2008). Another possibility is that elevated rhizosphere effects on microbial biomass and C mineralization affected other aspects of microbial activity that were not measured in this study—for example N mineralization or phosphatase activity (Zhao et al. 2007). Finally, it is possible that ECM hyphae, which are known to produce lignolytic and proteolytic enzymes (Chalot and Brun 1998), were present in bulk soil, obscuring differences between rhizosphere and bulk soil.

Some of the complexity in rhizosphere effects across these ecosystems may be related to the interaction with the broad range of MAP across the gradient. Rhizosphere effects for GAP and NAG, two enzymes involved in the degradation of N-containing compounds, increased with rainfall (Table 2). In general, rhizosphere effects were not detectable in the most arid site (under pines), while they were present without exception in the wettest site under *Nothofagus* spp. (Tables 3 and 4). In pot experiments with herbaceous species, rhizosphere effects on decomposition have been greatest in high-moisture soils (Dijkstra and Cheng 2007; Dijkstra et al. 2010) and most prior studies of rhizosphere effects in forest ecosystems have taken place under enriched CO_2 and ample water availability (for example, Ross et al. 2006; Phillips et al. 2011, 2012). In our study, changes in water availability across our precipitation gradient likely resulted not only from changes in precipitation, but also from changes in soil type. In the two driest sites, soils with relatively coarse texture may have had diminished water retention capacity compared to the three wetter sites, further reducing water availability. In the three wettest sites, the andic properties of soils (Broquen et al. 2005) may have further increased water retention and availability.

Table 5 Comparison of slopes of microbial organic C acquisition (ln(BG)) to microbial organic N acquisition (ln(GAP + NAG)), by field season and microsite, with 95% confidence intervals. Lower and upper refer to lower and upper confidence intervals

		Sites with native vegetation			Pine plantations		
season	microsite	slope	lower	upper	slope	lower	upper
summer 2010	rhizosphere	1.08	0.52	2.26	0.68	0.33	1.40
	bulk	1.12	0.52	2.41	0.66	0.35	1.24
	general site	1.14	0.42	3.07	0.83	0.55	1.25
summer 2009	general site	0.87	0.36	2.08	1.06	0.55	2.07

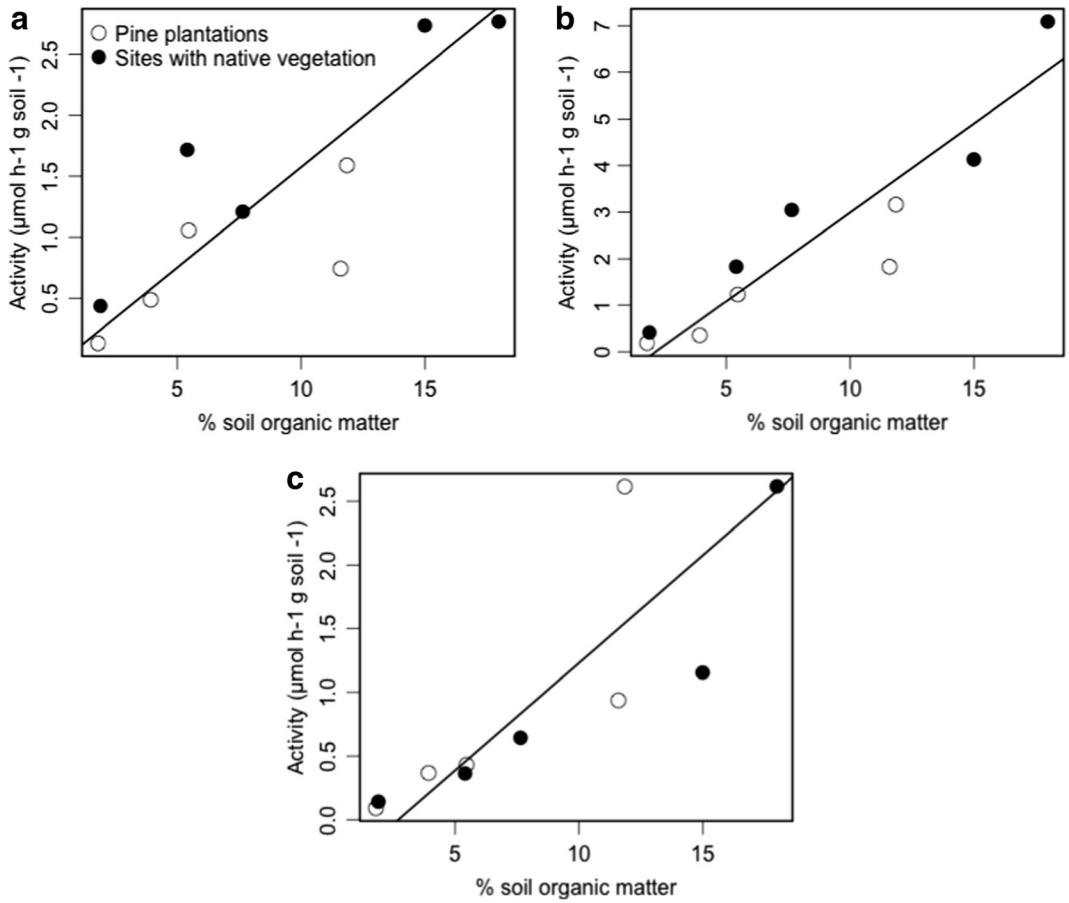

Fig. 4 Relationship between soil enzyme activities and soil organic matter. **a** BG activity. $y = 0.165x - 0.071$, $r^2 = 0.72$, $p < 0.005$. **b** GAP activity. $y = 0.381x - 0.816$, $r^2 = 0.84$, $p < 0.001$. **c** NAG activity. $y = 0.169x - 0.456$, $r^2 = 0.71$, $p < 0.005$

Low water availability in arid ecosystems could directly affect rhizosphere effects through impacts on root exudation, microbial activity, or solute diffusion in the soil. When C gain is restricted by water availability, producing root exudates to increase nutrient acquisition may not confer any advantage. Furthermore, low soil moisture may limit microbial activity in the rhizosphere, regardless of the presence of root exudates. It has also been proposed that exudates diffuse more slowly away from roots in dry soil, increasing the chances that they may be re-absorbed by the roots themselves (Jones and Darrah 1993). An alternative or complementary explanation is that precipitation effects on soil organic matter drive increased rhizosphere effects at higher levels of precipitation, as soil organic matter increases with precipitation in these sites (Hess and Austin 2014). The benefit to plants for investing resources in mining organic nutrients might be higher when organic nutrient stores are coupled with higher nutrient demand from increased net primary productivity (Araujo 2012). The trend of increasing rhizosphere effects with precipitation could also have been influenced by changes in soil properties along the precipitation gradient (Broquen et al. 2005), with higher concentrations of short-range order minerals (e.g. allophane, imogolite) in wetter sites potentially adsorbing and stabilizing rhizosphere enzymes (Matus et al. 2014).

Controls on soil enzyme activity

It is thought that soil enzyme activity is driven by a balance between soil substrate and nutrient supply, and microbial nutrient and energetic demand, although the complexity of these interactions remains poorly understood (Allison et al. 2010). Strong correlations between soil enzyme activities and C pools observed in this study support the idea that the quantity of soil organic matter and microbial biomass

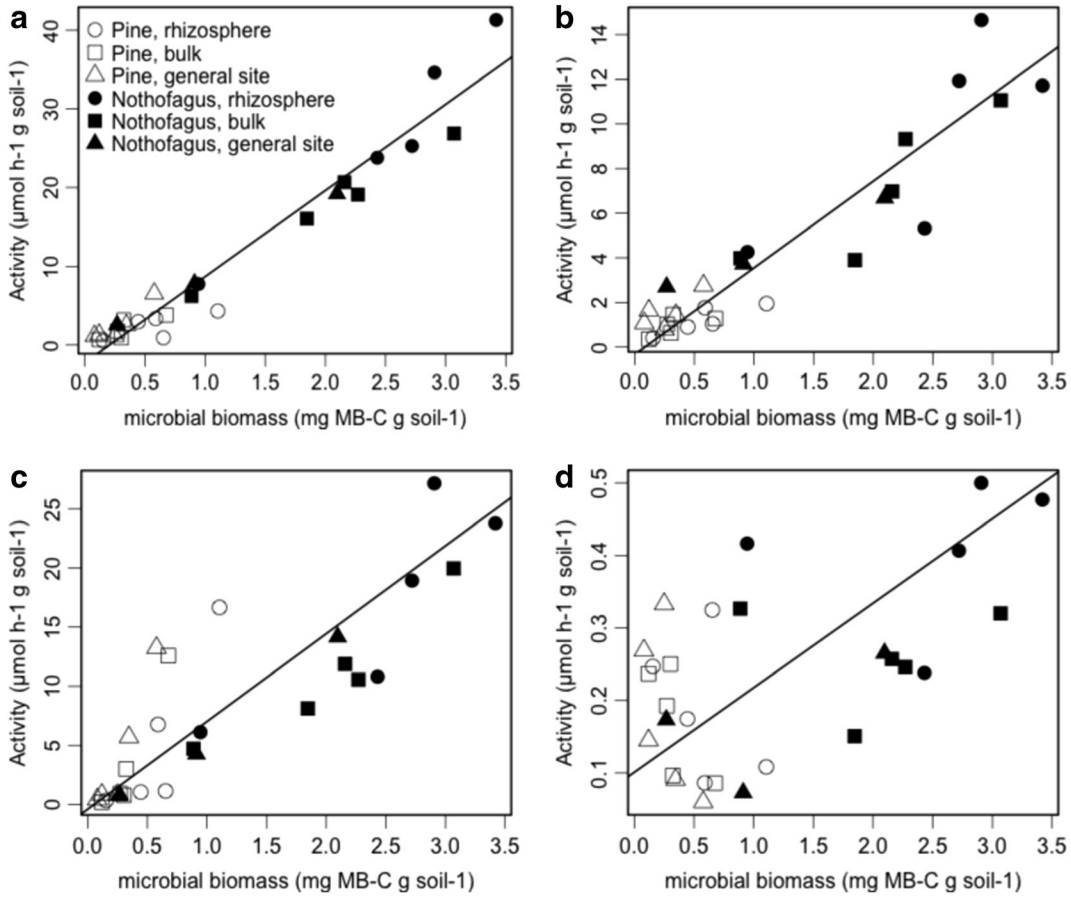

Fig. 5 Relationship between soil enzyme activities and MB-C. **a** BG activity. y = 10.938x – 2.236, r^2 = 0.95, $p < 0.001$. **b** GAP activity. y = 3.88x – 0.333, r^2 = 0.87 , $p < 0.001$. **c** NAG activity. y = 7.42x – 0.402, r^2 = 0.75, $p < 0.001$. (d) POX activity. y = 0.117x + 0.101, r^2 = 0.31, $p < 0.005$

are direct proximate controls on enzyme activity. Other studies have encountered similar relationships between some soil enzyme activities and organic matter, both within and across biomes (Sinsabaugh et al. 2005, 2008; Talbot et al. 2013). Sinsabaugh et al. (2008) found correlations between several hydrolase activities, including BG and NAG, and soil organic matter concentrations in a global dataset comprising a range of biomes. Sinsabaugh et al. (2005) found that changes in a composite factor of soil enzyme activity were proportional to changes in soil organic matter following simulated N deposition in forest ecosystems. Andersson et al. (2004) found a correlation between MB-C and chitinase activity (but not cellulase activity) in European forest soils experiencing different levels of N deposition. Our work supports the idea that relationships between hydrolytic enzymes mediating C- and N-degradation and soil C pools are generally consistent, even in human-engineered ecosystems.

In contrast to the hydrolytic enzymes, POX showed a negative relationship with MAP (in pine plantations), no relationship with SOM, and MB-C explained less of its variation. These findings are consistent with a review of POX activity across ecosystems (Sinsabaugh 2010). Phenol oxidase activity is often uncorrelated with, and thus may be controlled by different factors than, hydrolytic enzyme activity (Sinsabaugh 2010). For example, phenol oxidases are less stable in the environment than hydrolases, and particularly so in the presence of organic matter (such as at the high end of our precipitation gradient) (Sinsabaugh 2010). In addition, in arid ecosystems, high pH, low soil organic matter, and dry conditions may stabilize these enzymes (Sinsabaugh 2010).

The consistent relationships between microbial organic C and N acquisition, independent of climate or vegetation, support the idea of a relatively constrained global stoichiometry in terrestrial ecosystems, and a limited capacity of microbial communities to alter stoichiometric relationships of C and N acquisition activities in response to environmental resource availability (Fig. 3, Table 5, Sinsabaugh et al. 2009). Given the dramatic range of dominant vegetation and climate represented in our sites, and the different microsites within the soil environment that we sampled, we might have expected some variation in microbial community composition and/or environmental resource availability, and thus some variation in the relationship between C- and N-acquiring enzymes. However, soil microbial communities in all sites and microsites appear to behave according to similar stoichiometric relationships, with quite constant scaling relationships between microbial organic C and N acquisition (Table 5).

Implications of exotic pine afforestation for ecosystem functioning

The impacts of afforestation on soil C stocks has been the focus of a number of studies, and contradictory results have been reported (Guo and Gifford 2002; Paul et al. 2002; Berthrong et al. 2009; Eclesia et al. 2012). However, when afforestation with pine species is examined separately from that with other tree species, it is generally observed that soil C and N decline compared to non-afforested sites (Paul et al. 2002; Berthrong et al. 2009; Hess and Austin 2014). In these study sites specifically, we found significantly reduced soil C and N pools in pine plantations compared to sites with native vegetation in a previous study (Hess and Austin 2014). One explanation for this reduction in soil organic matter is that slow litter decomposition in pine plantations (Gholz et al. 2000, Araujo and Austin 2015) results in reduced C inputs to the soil. Another possible explanation suggested by our data is that elevated C mineralization in the rhizosphere of pines accelerates SOM decomposition at the ecosystem scale.

On the whole, rates of all microbial processes we measured were much lower in pine plantations than under stands of Nothofagus *spp*. across a wide range of climatic regimes. Declines in soil organic matter (Hess and Austin 2014) were mirrored by declines in soil microbial activity, including enzyme activity involved in C and N cycling. This suggests that in pine plantations, reductions in soil organic matter and microbial biomass slow microbial and enzyme activity, ultimately slowing microbe-mediated C and N cycling. While other studies have found inhibitory effects of afforestation with *Pinus* spp. on soil microbial biomass and C mineralization rates relative to native vegetation (Ross et al. 1999, 2002; Chen et al. 2000; Scott et al. 2006; Macdonald et al. 2009), we directly link changes in soil enzyme activity to changes in soil C pools, including microbial biomass. The reduction in potential soil N availability observed in this study correlated to reductions in soil C pools should be considered for its implications for the potential for long-term C accretion in these plantation forests.

Conclusions

The effects of afforestation on biogeochemical cycling depend on the planted species (Berthrong et al. 2009), climatic factors (Hess and Austin 2014), and original vegetation prior to afforestation (Bárcena et al. 2014; Araujo and Austin 2015). This study highlights that overall reductions in soil organic matter pools as a result of afforestation led to predictable reductions in extracellular enzyme activity based on globally robust estimates of the relationship between soil organic matter and nutrient acquisition by soil microbes (Sinsabaugh et al. 2008). While we did not find elevated rhizosphere effects for enzyme activities in pine species compared to native tree species, rhizosphere effects for enzymes involved in the acquisition of N increased with MAP, demonstrating the importance of water availability as a direct or indirect control on plant investment in N acquisition. Finally, we found consistent relationships between enzymes involved in the degradation of C-containing compounds and those involved in the degradation of N-containing compounds across sites and microsites, suggesting a limited capacity of microbial communities to alter stoichiometric relationships of C and N acquisition activities. The potential negative impacts of afforestation on N cycling should be considered in evaluating the long-term potential for C sequestration in these human modified ecosystems.

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