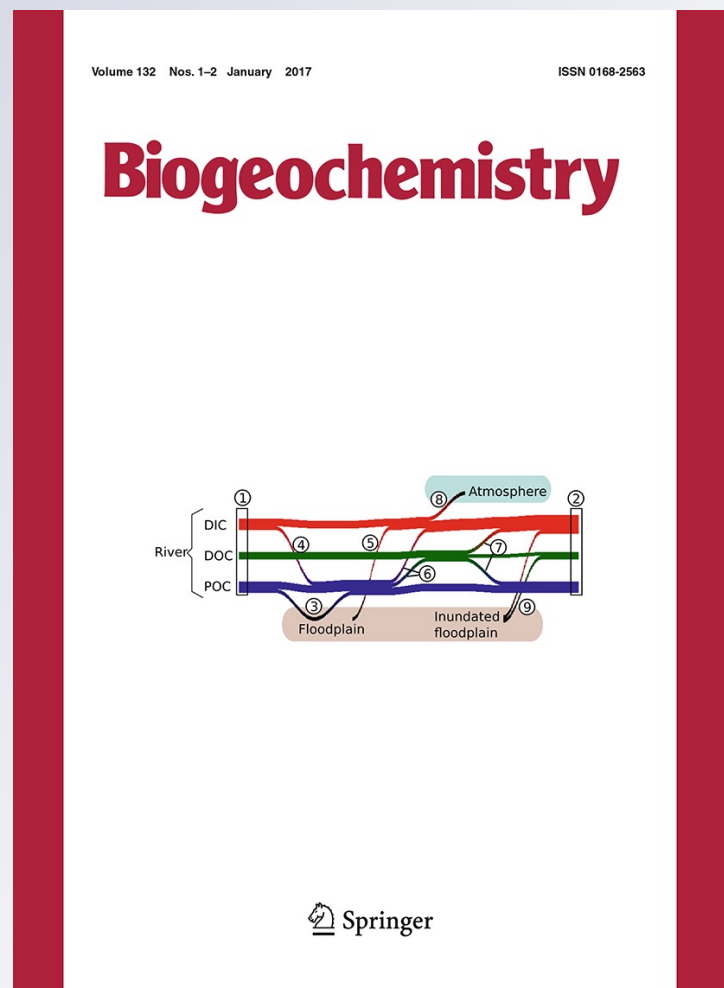# Biological nitrogen fixation in a post-volcanic chronosequence from south-central Chile

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Biological nitrogen fixation in a post-volcanic chronosequence from south-central Chile

Cecilia A. Pérez · Frank M. Thomas · Wladimir A. Silva · Rodrigo Aguilera · Juan J. Armesto

Abstract Biological nitrogen fixation is a key ecosystem function incorporating new nitrogen (N) during primary successions. Increasing evidence from tropical and northern temperate forests shows that phosphorus (P) and molybdenum (Mo) either alone or in combination limit the activity of free-living diazotrophs. In this study, we evaluated the effects of Mo, P, and carbon (C) addition, either singly or in combination, and moisture, on diazotrophic activity in a post-volcanic forest chronosequence in south-fentral Chile. Diazotrophic activity, both free-living (associated with fine litter) and symbiotic (associated with the moss *Racomitrium lanuginosum* and the cyanolichens *Pseudocyphellaria berberina* and *P. coriifolia*), was evaluated by incubation of samples and subsequent acetylene reduction assays conducted in the field and laboratory, in winter, spring and autumn of two consecutive years. Results showed that diazotrophic activity varied with the season of the year (lowest during the drier spring season), successional stage (highest in the maximal stage), and N-fixer community type (highest in symbiotic diazotrophs). In general, C+P+Mo limitation was documented for heterotrophic diazotrophs and P+Mo limitation for symbiotic diazotrophs. Limitation of diazotrophic activity was not associated with soil nutrient and C status in the chronosequence. Strong inhibition of diazotrophic activity by high N addition and by low moisture suggests that reductions in precipitation expected for south-central Chile under climate change, as well as increasing inputs of reactive N from atmospheric deposition due to increasing use of N fertilizers, may drastically alter the composition and functional role of cryptogamic assemblages in native forests.

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Introduction

Catastrophic disturbances such as lava flows and glacial retreat generate denuded substrates, which are rapidly colonized by pioneer organisms that can thrive under conditions of low nitrogen availability, such as

diazotrophic bacteria. These bacteria are able to fix atmospheric nitrogen (N) via biochemical reactions, facilitated by the nitrogenase enzyme complex. During early stages of primary succession in both post-volcanic and post-glacial substrates, diazotrophic bacteria are key players, by adding new N to the ecosystem and favouring soil development and the establishment of late successional species (Halvorson et al. 1992; Crews et al. 2000; Walker and Del Moral 2003; Menge and Hedin 2009). In temperate southern South America, volcanism has been a recurrent disturbance shaping the landscape and ecosystems during the entire Holocene and in recent historical times, associated with the 30 or so active, Andean volcanoes found along this latitudinal range. Given this unsettling geological scenario, understanding the constraints on the process of ecosystem recovery from severe disturbances becomes especially relevant.

Our knowledge of the drivers of nutrient and C limitation of biological nitrogen fixation (BNF) during the entire ecosystem development following catastrophic disturbances is still limited. Such understanding is globally relevant, especially for Southern Hemisphere temperate forests, which are subjected to much lower levels of atmospheric N deposition (Vet et al. 2014) than their northern hemisphere counterparts. In addition, during post-volcanic forest recovery in the southern Andes, strong seasonal and inter-annual variability in moisture levels affect soil microbial N transformations (Pérez et al. 2004, 2009a) and tree growth patterns (Pérez et al. 2009b; Walter et al. 2016). Vast regions of temperate forests in southern South America are now experiencing a strong precipitation decline, beginning in the past century (Quintana and Aceituno 2012), which may affect BNF rates during ecosystem recovery processes. To assess the response of BNF to these changing stresses, it is critical to understand how ecosystems recover from catastrophic disturbances, when biogenic elements are exhausted, and how these systems will respond to increasing anthropogenic fertilization and decreasing precipitation.

The nitrogenase enzyme complex includes an iron-sulphur and an iron-sulphur-molybdenum (Mo) protein that uses an ATP-activated electron transport chain to reduce atmospheric N_2 to ammonia (NH_3). Two alternative nitrogenase complexes have also been described: the vanadium-nitrogenase and the iron only-nitrogenase, which, in spite of being less efficient than the Mo-nitrogenase, can be relevant players in

substrates characterized by Mo limitation (Bellenger et al. 2011). To facilitate comparisons with other studies on temperate and tropical ecosystems, this study focuses primarily on the functioning of the Mo-nitrogenase (nitrogenase hereafter). Because the nitrogenase needs a very high activation energy, as at least 16 ATP molecules are hydrolysed for each mole of ammonia produced, increasing soil N availability tends to reduce biological N fixation rates (Paul and Clark 1989), thus giving a strong negative feedback on diazotrophic activity. Moreover, carbon (C)-rich substrates are needed as an electron source during reduction of N_2 (Cleveland et al. 1999). It is also well documented that light, temperature, and moisture have positive effects on BNF, especially in cryptogamic covers (Belnap 2001; Gundale et al. 2012; Rousk et al. 2013). N-fixing bacteria can follow two alternative pathways: they are either heterotrophic, free living on litter and soils, or part of symbiotic associations in cryptogamic flora and in nodulating legumes or even in non-legume vascular plants. This raises the question of whether the same factors controlling BNF apply to both N-fixing pathways.

A number of studies have investigated the nature of nutrient limitation on this key biogeochemical process. Most studies have been conducted in N-rich tropical forest soils (Vitousek and Hobbie 2000; Reed et al. 2007, 2013; Barron et al. 2008; Wurzburger et al. 2012) or in soils of temperate forests poorer in N (Silvester 1989; Pérez et al. 2008; Jean et al. 2013). According to studies in tropical soils, the main elements controlling the rates of heterotrophic BNF are phosphorous (P) on P-poor soils (Reed et al. 2007; Reed et al. 2011) and/or Mo, when soil P is more abundant (Barron et al. 2008; Wurzburger et al. 2012; Reed et al. 2013). The experimental addition of both P and Mo in combination had a greater stimulating effect on BNF than P alone (Vitousek and Hobbie 2000). In a similar experiment, added Mo enhanced BNF only when P was applied in a Mo-free fertilizer (Barron et al. 2008). Based on this new evidence, the authors argued for a "hidden" effect of Mo "contaminating" the P fertilizer, thus producing a positive effect on BNF when P was applied alone. Regarding symbiotic diazotrophs in both tropical and temperate forests, P fertilization and enhanced soil P availability had a positive effect on the abundance and growth of epiphytic cyanolichens (Benner and Vitousek 2012; Marks et al. 2015). In tropical forests of Costa Rica,

leaf C/N ratio correlated positively with BNF by foliar epiphylls (Cusack et al. 2009). Horstmann et al. (1982), on the other hand, documented that Mo had a positive effect on BNF by epiphytic cyanolichens in northern temperate ecosystems.

Most of our understanding about nutrient limitation to BNF comes from studies based on spatial gradients in soil fertility and focused mainly on either of the two BNF pathways: free-living or symbiotic diazotrophs (but see Yelenik et al. 2013). However, because nutrient and C limitation of BNF become more severe after major catastrophic disturbances, the study of nutrient limitation on BNF along volcanogenic soil chronosequences can enhance our understanding of the constraints of ecosystem recovery, where symbiotic diazotrophs play a key role in the incorporation of new nitrogen. For example, plant species such as *Coriaria* and *Gunnera*, both of which have symbiotic N fixers and appear in early stages of succession in glacier forelands of southern temperate ecosystems, play a key role in the recovery of organic matter and biogenic elements on denuded soil, with rates of N fixation of up to 20 kg ha^{-1} year^{-1} (Pérez et al. 2016). In later stages of ecosystem recovery after disturbance, when N availability becomes again limiting for plant growth, cryptogamic cover, including epiphytes and forest floor carpets, also play essential roles in N fixation in humid forests (Zackrisson et al. 2004; Lagestrom et al. 2007; De Luca et al. 2008; Menge and Hedin 2009; Pérez et al. 2014a). In southern temperate and Sub-Antarctic forests of South America, estimates of symbiotic BNF associated with cryptogamic carpets represented 25–50% of the total N fixed in advanced stages of succession (Pérez et al. 2009a, b). In a long-term post-volcanic chronosequence in the temperate region of Chile, both free-living and symbiotic diazotrophs presented a consistent pattern in the rates of BNF reaching higher values at the stage of maximal forest growth, and lower figures during both the progressive and retrogressive phases of ecosystem development (Pérez et al. 2014b), suggesting a similar nature of nutrient limitation for the different types of N-fixing communities.

The main objective of this work was therefore to investigate if there is a consistent limitation in the main controls of diazotrophic activity in free-living (associated with fine litter) and symbiotic (associated with lichens and mosses) diazotrophs along a post-volcanic ecosystem development sequence in the south-central region of Chile, focusing especially on the effects of nutrient, C and water limitation.

We addressed the following hypotheses about the controls of diazotrophic activity during succession:

(1) The addition of C, Mo and P to the various types of N-fixers in the long-term chronosequence should have a positive effect on nitrogenase activity, especially by enhancing diazotrophic activity during the progressive phase of soil development, when soil nutrient and C concentrations are lower.

(2) Experimental N addition should have no significant inhibitory effects on nitrogenase activity during the progressive phase, when low soil N availability limits plant growth.

(3) Predicted responses should be more pronounced for free-living N-fixers than for symbiotic ones, as the former are more responsive to changes in soil nutrient and C availability.

Materials and methods

Study sites

The present study was conducted on the eastern flanks of the Llaima volcano in the Conguillío National Park (38°41′S; 71°43′W), south-central Chile, within a relatively small area of about 90 km^2, where we find a mosaic of soils of varying age, reflecting a long history of volcanic disturbances. In this area, we defined a long-term, post-volcanic soil development chronosequence, where soil chemical properties, BNF, and denitrification patterns change significantly over the course of ecosystem development (Pérez et al. 2014b). From this previously established chronosequence, we selected three study sites, which represent three distinct successional stages of ecosystem development: (1) the progressive phase where plant establishment is the dominant process (substrate age 260 y BP), (2) the maximal growth phase, where forest vegetation has achieved maximum productivity (substrate age 3470 y BP), and (3) the retrogressive phase, where forest growth has been sustained for thousands of years (substrate age about 60,000 y BP). The prevailing climate of the study region is wet temperate with a pronounced Mediterranean influence, manifested in drier springs and summers. Records for 43 years from

a meteorological station located 22.5 km north of the study site indicate an average annual precipitation of 1945 mm and an annual average temperature of 8.6 °C. According to di Castri and Hajek ([1995]), 39% of the total annual precipitation falls during autumn (April-June), 38% during winter (July–September), 14% during the spring (September-December) and only 9% during summer (January-March). The dominant vegetation is mixed evergreen *Nothofagus-Araucaria* forests (Pérez et al. [2014b]). Canopy openness varied over the course of succession from 100% in the progressive phase, to 15.8% in the maximal phase, and to 7.1% in the retrogressive phase. Mean photosynthetically active photon flux density was derived from digital analyses (WinSCANOPY Pro 2009a software; Regent Instruments, Quebec, Canada) of hemispherical photos (cf. Walter et al. [2016]). During the growing season, it amounted to 1.25 ± 0.43 mol m^{-2} day^{-1} in the maximal and 0.50 ± 0.24 mol m^{-2} day^{-1} in the retrogressive phase below the canopy, and to 7.8 ± 0.9 mol m^{-2} day^{-1}, above the canopy.

Samples of N-fixer communities and element additions

In each study site, random samples of fresh litter and the dominant cryptogamic species, which presented symbiotic associations with cyanobacteria, were collected over two consecutive years during winter, spring and autumn 2013–2015. The following four types of N-fixer communities, belonging to either the free-living or the symbiotic pathway, were analyzed separately in this study: (1) free-living heterotrophs in the O_l soil horizon (recently fallen fine litter; consisting of mostly unbroken leaves and small twigs <2 mm of diameter) in each of the three sites, (2) symbiotic diazotrophs associated with the moss carpet of *Racomitrium lanuginosum* that dominates the biological soil crust in the progressive phase (with *Stigonema* and *Nostoc* as epiphylls), (3) epiphytic cyanolichen cover on trees and logs, including *Pseudocyphellaria berberina* with *Nostoc* (forming cephalodia in a green algal matrix), which are characteristic of the maximal successional stage, and (4) epiphytic cyanolichens, mainly *Pseudocyphellaria coriifolia* with *Nostoc* as photobiont, growing on tree trunks of the retrogressive phase. In the present study, the group 2) of epiphylls on the moss *Racomitrium* was included in the group of

true symbiotic diazotrophs such as the cyanolichens (groups 3 and 4), because they present a strong degree of association with the moss, in which the moss benefits from the cyanobacteria by taking up fixed nitrogen and the cyanobacteria take advantage by incorporating organic C from the moss (Rousk et al. [2013]).

In the three successional sites, samples of litter and cryptograms were homogenized as much as possible by hand, and then 6 g of cryptogams and 10 g of litter were placed in separate glass jars for incubations. Nutrients and C were experimentally applied in a 10 ml solution per jar, using concentrations in the high range of those that had produced significant effects on diazotrophic activity according to the literature (Reed et al. [2007]; Barron et al. [2008]; Reed et al. [2013]), and also affected diazotrophic activity in preliminary trials (data not shown). We therefore used the following chemical solutions and element concentrations: 2 mg Mo kg^{-1} were applied as Na_2MoO_4 (<5 mg kg^{-1} - PO_4^{3-}, final concentration <2 ppb P, Winkler, USA), 3 g N kg^{-1} were supplied as $(NH_4)_2SO_4$, 1 g of P kg^{-1} was added as KH_2PO_4 (Mo-free, Merck KGaA, Germany), and 6.3 g C kg^{-1} were added in the form of glucose. Experimental treatments were as follows: (1) one group of control samples, incubated at field moisture levels, without element additions; (2) a second group of control samples, adding de-ionized water (DIW), without element additions; (3) addition of N alone (as $(NH_4)_2SO_4$), (4) addition of P alone, (5) addition of Mo alone, (6) addition of C alone, (7) addition of Mo+P, (8) addition of C+Mo+P. Treatments 6 and 7 were applied using the same concentrations as the respective single-element treatments. The concentration of Mo added in experimental treatments was one order of magnitude higher than values of available Mo reported for Chilean volcanic Andisols (Vistoso [2005]; Vistoso et al. [2012]), and about the same concentration of total Mo as found in the litter sampled in the present study. P supply was two orders of magnitude higher than values of available P reported for soils of the same study sites (Pérez et al. [2014b]), and one order of magnitude higher than the total concentration of P found in litter samples. The addition of deionized water increased the water content of field samples from 51% to 78% on average depending on the season, N-fixer community type, and site. Seven replicates per treatment were used for a total of 56 samples per N-fixer community

type, making a total of 112 samples per successional site. These experimental procedures and subsequent acetylene reduction assays were conducted for two consecutive years during winter, spring and autumn 2013–2015.

Acetylene reductions assays

Samples from each N-fixer community and study site were incubated in hermetically closed glass jars containing a mixture of air and acetylene at 10% v/v. One additional sample per treatment was incubated without acetylene, as a control for basal levels of ethylene production. Diazotrophic activities were measured using the acetylene reduction assay (Myrold et al. 1999). This method was used based on the fact that the nitrogenase complex can also reduce acetylene to ethylene and that this latter product can be measured as an indicator of the activity of N fixers. After nutrient, water and C application, samples were allowed to equilibrate overnight. On the next day, acetylene was injected to the samples (see below) and the bottles containing samples were incubated in the field for up to 2 days in the case of litter and up to 8 h in the case of cryptogams. Additionally, in each season and at each site, random samples of each of the four N-fixer community types were taken for laboratory incubations in order to control for micro-environmental variation in the field. In the laboratory, mean temperature during the incubations was 23 °C and mean photosynthetically active photon flux density during the day was 0.17 mol m^{-2} day^{-1}.

Three gas samples per jar were taken periodically and injected into 4 ml Becton–Dickinson Vacutainers^{TM} for subsequent analyses. In the laboratory, gas samples were analyzed for ethylene production in a Shimadzu gas chromatograph GC-8AIF (Kyoto, Japan) equipped with a steel column filled with Porapak N (Supelco^{TM}, 1 m*1/4″ss, 80/100 mesh (Bellefonte, Pennsylvania, USA), and with an FID detector. Ethylene concentration in field and lab incubated samples was determined from a calibration curve by diluting a 100 ppm ethylene standard from Scotty^{TM} analysis gases (Bellefonte, Pennsylvania, USA). Acetylene reduction activity (ARA) was estimated from the slope of the linear fit of the ethylene production curve during the period of incubation in the jar headspace and referred to dry weight.

Chemical analyses of plants and soils

In May 2013, and at each successional site, random samples of surface soil (A$_h$), fine litter layer (O$_l$), and cryptogamic species (*Racomitrium lanuginosum*, *Pseudocyphellaria berberina,* and *P. coriifolia*) were taken for chemical analyses of element contents. Plant available P in soils was extracted through lactation by the Calcium-Acetate-Lactate method (CAL) and determined colorimetrically by the molybdenum blue method (Steubing and Fangmeier 1992). Samples of fresh soils were extracted in a 0.021 M KAl(SO$_4$)$_2$ solution (1:4). Ammonium and nitrate concentrations were measured by means of fractionated steam distillation (Pérez et al. 2004). Total C and N in plants and soils were analysed from ground samples by flash combustion in a Carlo Erba NA 2500 element analyser (Milan, Italy). Total P from soil samples and plant material was extracted with a concentrated sulfuric acid/water peroxide solution in a Hach Digesdahl digestor, (Loveland, Colorado, USA), and determined by colorimetry with the molybdenum-blue method. All analyses were conducted at the Biogeochemistry Laboratory, Pontificia Universidad Católica de Chile, Santiago. Total concentrations of Mo in soil and plant samples were determined in a graphite furnace AAS at the Geobotany Laboratory, Universität Trier, Germany, after extraction with nitric acid/water peroxide solution. The δ^{15}N isotopic ratios in samples of soil and plants were analyzed by mass spectrometry in a Thermo Delta V Advantage Isotope Ratio mass spectrometer (Thermo Finnigan, Bremen, Germany) at the Geobotany Laboratory, Universität Trier, Germany.

Statistical analyses

One-way ANOVAs and a posteriori Tukey's tests were used to evaluate differences in chemical properties of soils and plants among the three successional phases in the chronosequence. ARA data were box-cox transformed (Chen and Lockhart 1997) owing to the high frequency of null values. To test for the effects of sampling season, successional stage, and N-fixer community type on ARA in control samples incubated at field moisture levels, a two-way ANOVA for repeated measurements was applied. To assess element and water limitation on ARA, seasonal data were averaged to obtain an annual mean. P, Mo and C

limitation on diazotrophic activity for each successional site and N-fixer community used in the incubations was evaluated by running two-factor ANOVA (two levels: with and without addition of either P, Mo or C) and Holm-Sidak a posteriori tests for multiple comparisons. One-way ANOVA was used to evaluate water and N-$(NH_4)_2SO_4$ limitation on ARA followed by a posteriori Tukey's tests. Differences with respect to DIW controls were considered statistically significant at $P < 0.05$. The effect size of differences between treatment incubations and DIW controls was evaluated using Cohen's d index (Sullivan and Feinn 2012). Statistical analyses were conducted using R 2.5.1 software (R Development Core Team 2012).

Results

Chemical element concentrations of soils and N-fixers

Total concentrations of C, N, P, Mo and available P in soils from Conguillío Volcano were significantly lower in the progressive phase of post-volcanic succession than in any of the later phases in the chronosequence (Table 1). Total concentrations of N and P in litter increased from the progressive, maximal to the retrogressive phase of succession, but Mo and C concentrations in litter did not change across successional stages (Table 1).

The concentration of available N was highest in soils from the retrogressive phase (Table 1). The $\delta^{15}N$ ratio was significantly lower in soils and litter from the maximal phase of succession and, finally, soil C/N ratios in soils were similar at all sites regardless of successional stage, while the litter C/N ratio declined from the progressive to the retrogressive stage of succession (Table 1).

Among the cryptogamic N-fixers (Table 2), the moss *Racomitrium lanuginosum* from the progressive phase exhibited the lowest total N and P concentrations, as well as the minimum $\delta^{15}N$ ratios, but at the same time, this moss exhibited the highest concentrations of Mo and the largest C/N ratio. The cyanolichens *Pseudocyphellaria berberina*, which occurs in the maximal phase, and *P. coriifolia*, which occurs in the retrogressive phase, showed similar N concentrations. The former species displayed the lowest total concentration of Mo, while *P. coriifolia* showed the highest P concentration and the lowest C/N ratio among the investigated cryptogams.

Effects of successional stage, N-fixer community type and season on ARA

A repeated measurements analysis of variance revealed significant effects of successional stage, season and type of N-fixer community on the acetylene reduction rates of control samples incubated at field moisture levels, in addition to significant interaction effects among the three factors (Table 3). The highest diazotrophic activity was recorded in samples from the maximal phase of succession, while the lowest rates were recorded in the progressive phase

Table 1 Mean element concentrations (±1 SE), C/N ratios and isotopic ^{15}N signals in surface soils (A_h, $n = 6$) and litter layer (O_l, $n = 6$) from three stages of ecosystem development in Conguillío National Park, Chile

	Progressive		Maximal		Retrogressive	
	A_h	O_l	A_h	O_l	A_h	O_l
C (%)	0.41 ± 0.2b	50.1 ± 2.9a	11.37 ± 6.5a	48.3 ± 2.5a	13.89 ± 7.4a	46.9 ± 1.05a
N (%)	0.02 ± 0.01b	0.67 ± 0.14c	0.42 ± 0.2a	0.86 ± 0.07b	0.56 ± 0.3a	1.2 ± 0.2a
P (mg kg^{-1})	71.8 ± 20.9b	168.5 ± 21.7c	308.6 ± 97.0a	386.9 ± 41.2b	356.6 ± 80.0a	512.1 ± 88.6a
Mo (mg kg^{-1})	1.37 ± 0.34b	1.36 ± 0.24a	1.96 ± 0.48a	1.32 ± 0.17a	1.98 ± 0.33a	1.78 ± 0.07a
C/N (g g^{-1})	26.2 ± 14.4a	78.2 ± 17.3a	26.6 ± 4.3a	56.7 ± 3.8b	24.9 ± 0.8a	39.0 ± 5.1c
P_a (mg kg^{-1})	5.8 ± 0.7b	–	12.5 ± 4.0a	–	13.3 ± 1.9a	–
N_a (mg kg^{-1})	10.1 ± 5.4b	–	27.9 ± 10.4b	–	64.4 ± 11.1a	–
$\delta^{15}N$ (‰)	−1.86 ± 0.5a	−2.149 ± 0.553a	−3.78 ± 0.9b	−7.975 ± 0.246c	−1.61 ± 0.5a	−3.270 ± 0.566b

Different letters indicate significant differences among successional stages (Tukey's tests $P < 0.05$). P_a available P, N_a available N

Table 2 Mean element concentrations (± 1 SE), C/N ratios, and ^{15}N isotopic signal measured in the biomass of the dominant cryptogamic species ($n = 6$) known to have symbiosis with N-fixing bacteria in three stages of ecosystem development in Conguillío National Park, Chile

	Racomitrium lanuginosum	*Pseudocyphellaria berberina*	*Pseudocyphellaria coriifolia*
C (%)	34.6 ± 2.0c	50.4 ± 0.6a	40.6 ± 3.0b
N (%)	0.72 ± 0.03b	2.33 ± 0.15a	2.36 ± 0.19a
P (mg/kg)	366.2 ± 21.6c	631.6 ± 186.3b	1200.4 ± 202.5a
Mo (mg/kg)	4.34 ± 1.30a	0.74 ± 0.16c	1.78 ± 0.25b
C/N	48.23 ± 4.32a	21.67 ± 1.33b	17.25 ± 0.97c
δ^{15}N (‰)	-4.77 ± 0.16b	-3.13 ± 0.15a	-2.80 ± 0.07a

Different letters indicate significant differences among cryptogamic species (Tukey's tests, $P < 0.05$)

Table 3 Statistical parameters of two-way ANOVA for repeated measurements assessing the effect of the stage of ecosystem development, N-fixer community type (free-living and symbiotic), and season of the year and their interactions on acetylene reduction activity by diazotrophs measured in field incubations at the field moisture content

| Effect	SS	df	MS	F	P
Stage of ecosystem development	67.98	2	33.99	57.9	<0.0001
N-fixer community type	59.19	1	59.19	100.8	<0.0001
Stage of ecosystem development × N-fixer community type	31.96	2	15.98	27.2	<0.0001
Season	28.52	2	14.26	39.8	<0.0001
Season × stage of ecosystem development	14.89	4	3.72	10.4	<0.0001
Season × N-fixer community type	17.48	2	8.74	24.4	<0.0001
Season × stage of ecosystem development × N-fixer community type	14.07	4	3.52	9.8	<0.0001

($P < 0.0001$). Symbiotic diazotrophs (associated with cyanolichens) presented the highest ARA among all the substrates incubated ($P < 0.0001$). Regarding seasonal differences, pooling data from all sites, the lowest ARA was measured during spring incubations ($P < 0.0001$).

Effects of nutrient, C and water addition on ARA in heterotrophic diazotrophs

The addition of Mo to heterotrophic diazotrophs incubated in the field significantly increased ARA at the maximal stage of succession (Fig. 1a; Table S1a), with an effect size of 5.83 (Tab S2). The addition of C+P+Mo significantly enhanced ARA in the progressive stage of succession (Fig. 1b; Table S1b), with an effect size of 1.76 (Table S2), as well as in the maximal successional phase (Fig. 1b; Table S1b), with an effect size of 1.83 (Table S2). No effect of C, Mo and P either singly or in combination was present in the retrogressive phase (Fig. 1a, b). Water addition to the incubated samples, on the other hand, significantly decreased ARA in the maximal phase (Fig. 1c; Table S1c), with an effect size of -2.42 (Table S2). Finally, experimental addition of $(NH_4)_2SO_4$ significantly depressed ARA in the maximal and retrogressive phases (Fig. 1c; Table S1c) with an effect size of -2.47 and -3.56, respectively (Table S2).

In laboratory incubations, the addition of Mo to heterotrophic diazotrophs significantly increased ARA in the maximal successional phase (Fig. S1a; Table S1a), with an effect size of 2.4 (Table S2). In this successional phase, adding C alone enhanced ARA (Fig. S1b; Table S1b) as well, with an effect size of 1.65 (Table S2). Water addition significantly increased ARA in the progressive and retrogressive phases (Fig. S1c; Table S1c), with an effect size of 15.15 and 2.12, respectively (Table S2). In contrast, ARA was significantly inhibited by the addition of $(NH_4)_2SO_4$ in samples from all three successional
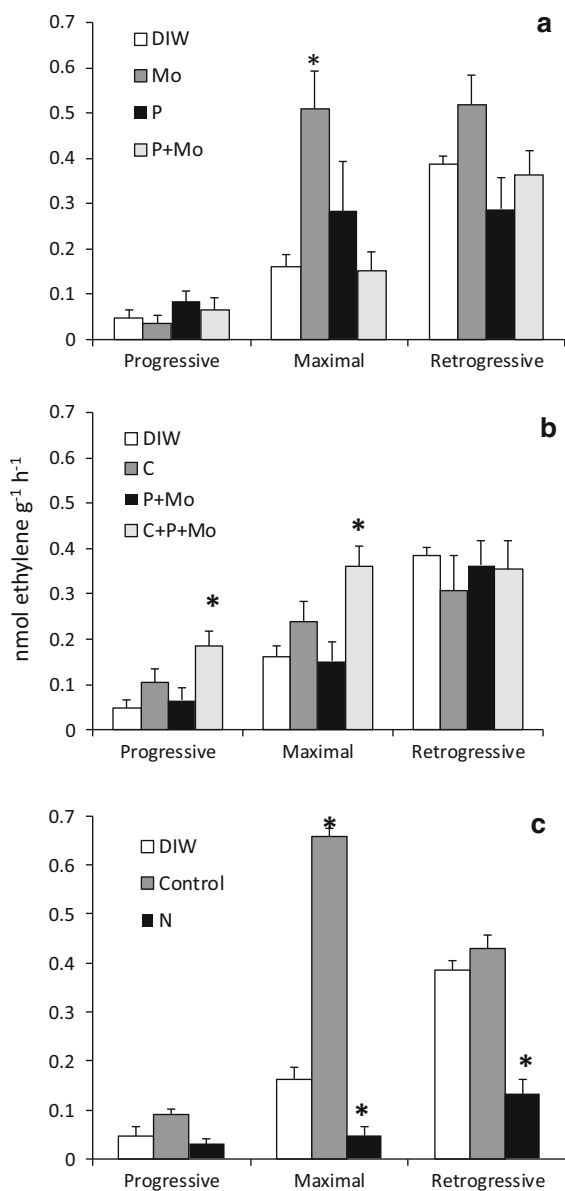
Fig. 1 Mean acetylene reduction rates (± 1 SE) by heterotrophic diazotrophs ($n = 6$) obtained from in situ incubations, in three stages of ecosystem development (progressive, maximal and retrogressive) in Conguillío National Park, Chile: **a** the effect of P and Mo addition, **b** the effect of C addition, and **c** the effect of water and N-$(NH_4)_2SO_4$ addition. *Asterisks* indicate statistically significant differences between element-addition treatments and the control with addition of de-ionized water (DIW) only

phases (Fig. S1c; Table S1c), with an effect size $\leq$ -30 (Table S2). Overall, ARA was higher in the laboratory than in field incubations.

## Effects of nutrients, C and water additions on ARA in symbiotic diazotrophs

Incubations of symbiotic diazotrophs in the field revealed significant enhancement of ARA by combined P+Mo addition to the lichen *P. coriifolia* of the retrogressive phase but the same combination of elements inhibited ARA in *P. berberina* of the maximal phase (Fig. 2a; Table S3a) with effect sizes of 2.02 and -4.27, respectively (Table S2). The addition of C had no significant effects on any treatment (Fig. 2b). Water addition significantly stimulated ARA in samples of *P. berberina* collected from the maximal successional phase and in samples of *P. coriifolia* from the retrogressive phase (Fig. 2c; Table S3c), with effect sizes of 7.49 and 12.19, respectively (Table S2). The addition of $(NH_4)_2SO_4$ significantly inhibited ARA in symbiotic diazotroph samples from all three stages of ecosystem succession (Fig. 2c; Table 3c), with an effect size of -5.7 or even more negative (Table S2).

In laboratory incubations, the addition of nutrients and C, applied either singly or in combination, had no significant effects on ARA (Fig. S2a, S2b; Table S3a, S3b). Water addition, in turn, increased ARA of cryptogamic covers from all successional phases (Fig. S2c; Table S3c), with an effect size of 2.3 or greater (Table S2). The addition of $(NH_4)_2SO_4$ inhibited ARA of cryptogamic covers regardless of origin (Fig. S2c; Table S3c), with effect sizes ≤ -7.8 (Table S2). Overall, ARA was higher in the field than in laboratory incubations.

Discussion

Diazotrophic activity along ecosystem development

In this study, we examined the activity of diverse diazotrophic systems in three stages of post-volcanic soil development, in different seasons of two consecutive years, and evaluated ARA responses to nutrient, C and water additions. The controls on diazotrophic activity, the main vector of new N to the ecosystem, can be critical regulators of forest transitions during succession following catastrophic volcanic disturbance. The highest rate of ARA (a proxy for BNF) on volcanic soils of Conguillío National Park was
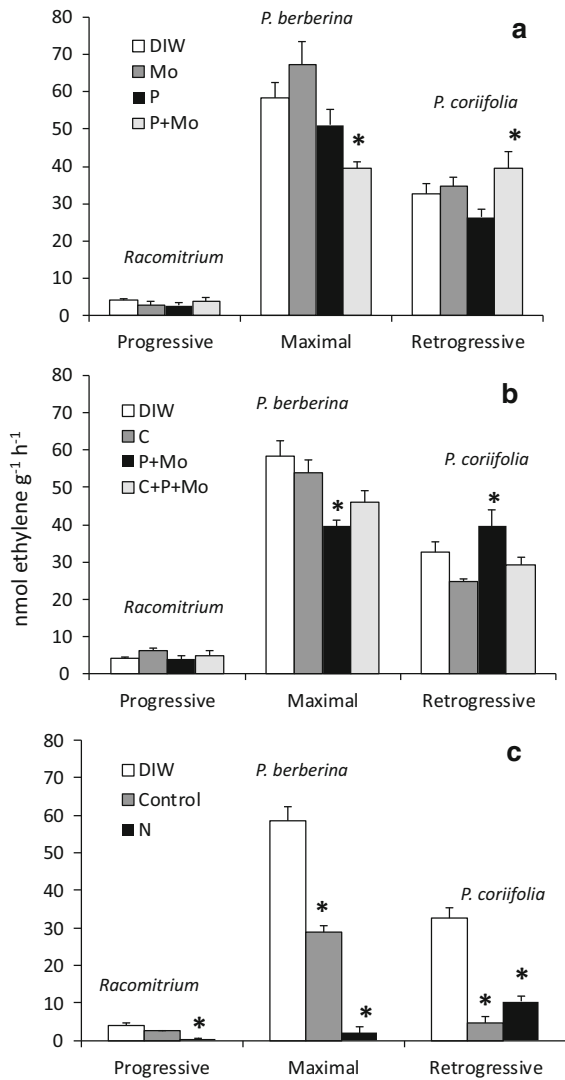
Fig. 2 Mean acetylene reduction rates (± 1 SE) by symbiotic diazotrophs ($n = 6$) measured during in situ incubations of cryptogamic species that are dominating in three different stages of ecosystem development (progressive, maximal and retrogressive) in Conguillío National Park, Chile: **a** the effect of P and Mo addition, **b** the effect of C addition, and **c** the effect of water and N-$(NH_4)_2SO_4$ addition. *Asterisks* indicate statistically significant differences between element-addition treatments and the control with addition of de-ionized water (DIW) only

recorded in N-fixers from the maximal phase of ecosystem development in the long-term chronosequence studied. High N fixation potential in this successional phase was linked to higher concentrations of total C, total N, Mo and P in soils than in the progressive phase of soil development. Soils of the maximal phase are more depleted in ^{15}N than soils

from the retrogressive phase, suggesting that the maximal phase is associated with a tighter N cycle (Pérez et al. 2014b). Under an open canopy, symbiotic autotrophs, such as epiphylls on *Racomitrium* from the progressive phase, did not exceed ARA of epiphytic cyanolichens. Incubations of *P. berberina* from the maximal phase, under higher canopy openness, showed higher ARA than *P. coriifolia* from the retrogressive phase, under lower canopy openness. This suggests that light may become a limiting factor for diazotrophic activity of cyanolichens under forest shade.

The lowest rates of ARA and hence biological N-fixation across all sites were recorded during the drier spring season. Moreover, the positive response of ARA to water addition (Fig. 2) and pronounced effect size (Table 2S) suggest that moisture supply is an important limiting factor for diazotrophic activity in temperate forests under Mediterranean-climate influence, as is the case beneath Conguillío volcano. Similar seasonal differences in the activity of free-living N_2 fixers have been reported for broadleaved evergreen forests in Chiloé Island (Pérez et al. 2004, 2009a, b) and for seasonal tropical forests (Reed et al. 2007).

As expected, diazotrophic activity was highest in symbiotic autotrophs, confirming previous evidence that cryptogamic species contribute importantly to the incorporation of new N to biomass in late stages of succession in forest ecosystems (Horstmann et al. 1982; Zackrisson et al. 2004; Gundale et al. 2009; Cusack et al. 2009). In the post-volcanic Conguillío chronosequence, the epiphytic cyanolichen *Pseudocyphellaria berberina,* covering tree barks during the maximal phase of forest succession, produced the highest ARA rates, up to 60 nmol of ethylene g DW^{-1} h^{-1} during winter months, which scaled up to an annual estimate of 3 kg N ha^{-1} (Pérez et al. 2014b). However, declining trends in precipitation in south-central Chile will have drastic effects on N-fixation provided by the cyanolichen species.

Nutrient, C and water limitation of diazotrophic activity

Similar to previous findings for tropical and northern temperate forests (Wurzburger et al. 2012; Jean et al. 2013), diazotrophic activity by free-living diazotrophs was enhanced in the present study when all critical

elements are added as C+P+Mo in field incubations, in the progressive and maximal phase of post-volcanic succession and plus Mo alone in the latter phase. Therefore, C and nutrient limitation to heterotrophic diazotrophic activity is confirmed in southern temperate forests as well. Moreover, water addition to incubated samples from the maximal successional phase slowed down diazotrophic activity, presumably because of an efflux of litter C in the form of DOC leachate (Belnap 2001). Addition of water increased soil water content from 61 to 78%, which probably did not create anoxic conditions that otherwise could have explained ARA reductions. In contrast to the effects of C, Mo, and P supplied in combination, we detected no effect of adding P alone on ARA by free-living diazotrophs in the field, even in the earliest successional phase, where concentrations of total and available soil P were the lowest in the chronosequence. Likewise, in lowland old-growth evergreen rainforest in Chiloé Island, we observed a lack of response to increasing doses of P addition by heterotrophic diazotrophs (Pérez et al. 2008). Studies in P-poor tropical soils, however, reported positive effects of P on N fixation by free-living bacteria, but only when added in combination with Mo (Wurzburger et al. 2012). They attributed their result to the fact that Mo is strongly fixed in soil organic matter, thus decreasing its overall availability relative to phosphate.

In summary, the results only partially support our Hypothesis 1, in the sense that we did find evidence for Mo and C+P+Mo limitation on free-living diazotrophic activity even during the maximal phase of post-volcanic ecosystem development, which is characterized by higher concentrations of total soil C, P and Mo than in the progressive phase, evidencing C and nutrient limitation of free-living diazotrophs as well. Lack of correlation between nutrient limitation of free-living N_2 fixers and soil contents of either P or Mo has also been documented for northern temperate forests in Canada (Jean et al. 2013). These line of evidences shows a more spontaneous and opportunistic response to nutrient and C additions of free-living diazotrophs rather than a preadaptation to site conditions.

In agreement with our Hypothesis 2, N addition as $(NH_4)_2SO_4$ inhibited nitrogenase activity in field incubations of heterotrophic diazotrophs in the maximal and retrogressive stages of succession at higher

N, but not in the progressive phase at lower N availability in soils. A similar inhibitory effect of N addition has been reported for moss-cyanobacteria carpets in boreal forests (Zackrisson et al. 2004; De Luca et al. 2008; Ackermann et al. 2012; Gundale et al. 2013). Sulfate has been documented to have negative effects on N fixation by planktonic cyanobacteria, because of the competitive displacement of molybdate (Marino et al. 2003). Therefore, in the present study, ammonium-sulphate addition could have displaced available Mo from the substrate, acting synergistically with N addition, to strongly inhibit diazotrophic activity.

In tropical old-growth forests in Hawaii, chronic N additions significantly inhibited free-living diazotrophs (Crews et al. 2000). In the present study, N was applied in a short pulse (estimated as ca. 15 kg ha^{-1} year^{-1}), one order of magnitude higher than annual rates of total N deposition in the study region (1–2 kg N ha^{-1} year^{-1}; Vet et al. 2014) and about two and a half times the total N inputs via BNF to these southern forests (Pérez et al. 2014b). We can expect therefore that under chronic N deposition, there will be dramatic shifts in cryptogamic species assemblages leading to an over-representation of species lacking N-fixing symbioses, especially in south-central Chile, where N-fertilizers are under increasing use on farms and pastures (FAO 2015).

Field incubations of symbiotic diazotrophs in our study sites showed an enhancement of ARA after P+Mo addition to *P. coriifolia* from the retrogressive phase of succession, which confirmed the assumption that these elements act together to enhance nitrogenase activity also in symbiotic diazotrophs. However, P+Mo addition to *P. berberina* samples from the maximal successional phase produced the opposite effect. Such contrasting effects of P+Mo addition to different substrates including free living diazotrophs has also been observed in cold temperate forests of Canada (Jean et al. 2013). Negative effects of P+Mo addition in some cases in the chronosequence studied might be explained by the fact that the molybdate anion can be a competitive inhibitor of sulfate reducing N-fixers in different environments (Oremland and Capone 1988; Desai et al. 2013). For sulfate respiring bacteria, it has been documented that Mo addition leads to both the formation of adenosine-phosphomolybdate (instead of adenosine-phosphosulfate used in chemical reduction steps) and the

depletion of ATP (Oremland and Capone 1988), which could be exacerbated under P addition. This biochemical mechanism may explain why the addition of P and Mo applied together to field incubated samples inhibit ARA. In the case of *P. berberina*, sulfate-respiring diazotrophic bacteria probably colonize cyanolichens in addition to *Nostoc*, either as epiphylls or endophytic. This assumption is based on the growing evidence that shows a high diversity of both pro- and eukaryotic algae and bacterial assemblages colonizing a single lichen (e.g. Bates et al. 2011; Park et al. 2015) and moss species (Park et al. 2013; Vile et al. 2014).

In summary, contradicting our Hypothesis 3, field incubations of symbiotic diazotrophs did show significant responses to addition of P+Mo, either positive or negative, depending on the cyanolichen species. Again, as in the case of free-living diazotrophs, such limitation was not related to low P or Mo concentrations in the lichen tissue. We also observed that ARA of symbiotic diazotrophs was reduced by one order of magnitude in laboratory assays compared to the field. We interpret these results as an indirect effect of lower light availability under laboratory conditions, but this effect was not evident in free-living diazotrophs.

Nutrient and C limitation of diazotrophic activity in relation to other forest ecosystems

The present study of southern temperate forests shows that field estimates of ARA for free-living diazotrophs (0.06–1.6 nmol ethylene $g^{-1} h^{-1}$) are within the range of estimates reported for lower latitude, warmer and wetter tropical forests (0.2–16.4 nmol ethylene $g^{-1} h^{-1}$, Barron et al. 2008; Cusack et al. 2009; Wurzburger et al. 2012). Lower rates of ARA are reported for high latitude northern temperate forests in Canada (0.01–0.7 nmol ethylene $g^{-1} h^{-1}$, Jean et al. 2013). Total soil concentrations of Mo found in the present study (1.37–1.98 mg kg^{-1}) were in the upper range of values known for northern temperate forests (0.535–1.25 mg kg^{-1}, Jean et al. 2013) and lower than the range of values reported for tropical forests (3.4–7.2 mg kg^{-1}, Reed et al. 2013). In contrast, Mo concentrations in the litter layer of southern forests reported in the present study (1.32–1.78 mg kg^{-1}) were higher than the values for northern temperate (0.047–0.338 mg kg^{-1}, Jean et al. 2013) and tropical forests (0.053–0.45 mg kg^{-1}, Reed et al. 2013). Higher Mo content in litter from southern temperate forests in this

study can explain the similar rates of ARA compared to tropical forests. Moreover, available Mo contents in volcanic soils of the study region (9–22 ppb, Vistoso et al. 2012) are similar to those reported for northern temperate regions (4–52 ppb; Jean et al. 2013), but higher than values reported by Wurzburger et al. (2012) for tropical soils (0.3–2 ppb). Although high Mo availability in soils and total Mo in the litter layer of these forests could suggest little or no Mo limitation, we still observed a positive response of heterotrophic diazotrophs to Mo addition, when applied either alone or in combination with C and P. The total P concentrations in soils (71.8–356.6 mg kg^{-1}) tend to be lower than in northern temperate forests from Canada (367–1871 mg kg^{-1}, Jean et al. 2013) and lower than in some tropical forests (388–557 mg kg^{-1}, Reed et al. 2013). Litter P concentrations in the present study (168–512.1 mg kg^{-1}), on the other hand, do not differ from values for tropical forests (160–844 mg kg^{-1}, Reed et al. 2013) and are lower than values for northern temperate forests (701–1488 mg kg^{-1}, Jean et al. 2013). Although low concentrations of total soil P suggest a high probability of P limitation of ARA, we did not observe a positive response of free-living diazotrophs to P addition in any stage of succession, except when added in combination with Mo and C.

For symbiotic diazotrophs, the range of ARA values (across species at field moisture content) in the present study (1.9–29.1 nmol ethylene $g^{-1} h^{-1}$) approaches high values reported by Cusack et al. (2009) for cryptogamic cover in tropical forests (0.01–12 nmol ethylene $g^{-1} h^{-1}$). However, the highest field rates of ARA were measured in the cyanolichen *Lobaria pulmonaria* from northern temperate forests, reaching up to 12,330 nmol ethylene $g^{-1} h^{-1}$ (Horstmann et al. 1982). In tropical montane forests of Hawaii, N-fixing cyanolichens showed a positive response to P addition at a rate of 100 kg P ha^{-1} $year^{-1}$ (Benner and Vitousek 2012). Such effect of P addition was not detected in the present study, except when P was added with Mo and C together at a rate of 5 kg P ha^{-1} $year^{-1}$. We infer from this result that southern temperate cyanolichens must have significantly lower P requirements.

Conclusions

When comparing the nature of nutrient limitation to diazotrophic activity, we found that C limitation had a

positive effect on heterotrophic diazotrophs when added together with P and Mo; because C, provided under natural conditions by the decomposing organic matter on the forest floor, is the diazotrophs' main source of electrons for N_2 reduction to NH_3. On the other hand, C addition had no effect on symbiotic diazotrophs, suggesting that enough C for the metabolism of the blue-green algae is fixed via photosynthesis. The combined addition of P and Mo had a stronger effect on diazotrophic activity which was either positive or negative, depending on the cyanolichen species, which in turn may be affecting the functional groups of bacterial assemblages in the symbiotic association as discussed above. N inhibition of diazotrophic activity was observed in both symbiotic and free-living diazotrophs across ecosystem development, confirming the strong negative feedbacks mechanism regulating N_2 fixation. On the contrary, moisture had a positive effect only on diazotrophic activity of epiphytic cyanolichens. In fact epiphytic lichens, as poikilohydric organisms, rapidly change their water content, which is not the case in the decomposing litter layer on the forest floor, with a larger capacity to retain water. In fact, in the litter layer the addition of water had a negative effect on free-living diazotrophs (Fig. 1c). Moisture levels and light intensity represent key limitations to symbiotic diazotrophs but no such response was found in free-living diazotrophs, whose ARA was not affected by the lower light availability under laboratory condition. Similar inconsistent response to nutrient addition in free-living and symbiotic diazotrophs has been reported in post-fire communities, where free-living diazotrophs increased N fixation after P addition, while symbiotic association in *Ceanothus* did not (Yelenik et al. 2013).

Regarding the nature of nutrient and C limitation in temperate forests, our data indicates that this limitation is not a direct consequence of soil nutrient and C status, which changes with the stage of post-volcanic ecosystem development. Responses of diazotrophic activity to C, nutrient and water supply are highly complex and dynamic, probably influenced by the diverse assemblages of diazotrophic bacterial communities and their various metabolic pathways (Riederer-Henderson and Wilson 1970; Vile et al. 2014), including diverse types of the nitrogenase enzymes not restricted to the Mo-, V- or Fe-nitrogenases. It has recently been shown that a CdS-nitrogenase can work even without requiring

ATP as a source of electrons for reducing N_2 to NH_3 (Brown et al. 2016). Consequently, we are only beginning to understand the variety of mechanisms that can explain the outcomes of experimental element additions to different types of microorganisms responsible for biological N inputs to ecosystems, and how they change through ecosystem development, converging finally to a similar pattern of BNF, driven by carbon, water and nutrient limitation.

Strong inhibition of diazotrophic activity by high N addition and by low moisture suggests that reductions in precipitation expected for south-central Chile under climate change, as well as increasing inputs of reactive N from atmospheric deposition due to increasing use of N fertilizers by land use change to pasture and agricultural lands, may drastically alter the composition and functional role of cryptogamic assemblages in the surrounding native forests.

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