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Source: Arctic, Antarctic, and Alpine Research, 49(1) : 29-42

Published By: Institute of Arctic and Alpine Research (INSTAAR), University of Colorado

URL: <https://doi.org/10.1657/AAAR0016-014>

# Limitations and relevance of biological nitrogen fixation during postglacial succession in Cordillera Darwin, Tierra del Fuego, Chile

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## ABSTRACT

We tested the main hypothesis that nutrient accumulation during late stages of postglacial succession would decrease nutrient limitation of diazotrophic activity. We tested this hypothesis by adding carbon (C), phosphorus (P), and molybdenum (Mo) independently or in combination, and nitrogen (N) only to symbiotic, epiphylls on bryophytes, and free-living diazotrophs in three stages of glacier foreland succession in Cordillera Darwin (55°S), southern South America. Experiments were run in spring 2013 and 2014 and in autumn 2015. Diazotrophic activity (DA) was assessed by the acetylene reduction assay. Results showed no effect of C, P, or Mo added either singly or in combination in the spring incubations. During autumn, DA was enhanced by adding a mix of C, P, and Mo to the symbiotic N<sub>2</sub>-fixing *Gunnera magellanica* from young successional sites, while in the late successional sites, adding C and Mo alone to the diverse bryophyte carpet on the forest floor enhanced DA. Nitrogen added as ammonium sulfate had a strong negative effect on N<sub>2</sub> fixation by free-living diazotrophs in the spring and autumn samples from the late successional site, in the bryophyte carpet from the early successional site (autumn), and in *Pseudocypbellaria freycinetii* of the midsuccessional site (spring). As in other high-latitude biomes, symbiotic and epiphyllous associations and free-living diazotrophs play a crucial role in the incorporation of new N to postglacial subantarctic forest ecosystems, especially in recently exposed substrates that are strongly limited by nutrient availability in soils. The increasing rates of glacier melting in southern South America is exposing new substrates to microbial colonization, including diazotrophic bacteria. In this environment, largely free of reactive N from atmospheric sources, new ecosystems are rapidly developing on deglaciated surfaces, provided that key elements such as Mo and P and C are present in the substrates.

## INTRODUCTION

In Cordillera Darwin ice fields, subantarctic region of Chile, rapid glacial retreat is more intense in the north-facing than in the south-facing slopes (Melkonian et al., 2013). Loss of ice cover produces a denuded rocky substrate, depleted of soil organic matter and nitrogen, although phosphorus may be available in P-rich surface

minerals (Richardson et al., 2004; Parfitt et al., 2005). However, some recently exposed substrates in the subantarctic region of Chile may also be P-poor when primary minerals such as granite are predominant in the substrate (Pérez et al., 2014). In such cases, ecosystem development is initiated by organisms that can capture N<sub>2</sub> from the atmosphere and are able to mine essential elements from denuded substrates (Chapin et al.,

1994; Hobbie et al., 1998). Biological  $N_2$  fixation is a key process in primary succession, carried out by either free-living bacteria (e.g., heterotrophic bacteria depending on carbon-rich substrates such as decomposing litter), symbiotic or epiphyllous prokaryotes, denominated diazotrophic bacteria (that feed on  $N_2$ ). In Arctic and subarctic ecosystems, cyanobacteria occurring as epiphylls on bryophytes, or conforming symbiotic associations in lichens, are the major providers of new N to the ecosystems (e.g., Solheim and Zielke, 2002; Rousk and Michelsen, 2016).

The nitrogenase enzyme complex in these microorganisms can be a highly efficient molybdenum (Mo)-nitrogenase, an iron-only nitrogenase or a vanadium (V)-nitrogenase. As Mo is the least abundant microelement in soils, it could be a possible limiting element for diazotroph activity (Bellenger et al., 2011). However, more recently, the nitrogenase form that uses V as a co-factor, which is a more abundant element in the Earth's crust, has been found in boreal cyanolichens (Hodkinson et al., 2014; Darnajoux et al., 2014). These various nitrogenase complexes need adenosine triphosphate (ATP) and electron sources (such as glucose carbon) for the energetically costly reaction that reduces  $N_2$  to  $NH_3$ . This reaction is in turn tightly regulated by a negative feedback mechanism dependent on available inorganic N in soils (Zuberer, 1998). For comparative purposes, in the present study we focused on the Mo-nitrogenase only (hereafter, nitrogenase). Because the nitrogenase complex can also reduce acetylene to ethylene, this product can be measured as a proxy for diazotrophic activity (DA).

Studies in Arctic and subarctic polar deserts and Arctic tundra have shown that seasonal increases in soil moisture associated with snowmelt can greatly enhance both free-living, symbiotic and epiphyllous DA (Alexander and Schell, 1973; Dickson, 2000; Zielke et al., 2002, 2005; Sorensen et al., 2006; Stewart et al., 2011; Rousk et al., 2015). Enhancement of DA by higher soil moisture levels has also been observed in boreal forests (Gundale et al., 2009; Jackson et al., 2011). Consequently, in high-latitude ecosystems, pronounced seasonality of abiotic variables such as moisture, light, and temperature are key drivers of diazotrophic activity. High diazotrophic activity was associated with peak moisture levels during the growing season after snowmelt, concomitant with higher temperatures and enhanced light conditions; however, DA also varies depending on  $N_2$  fixer community type (Alexander and Schell, 1973; DeLuca et al., 2002; Hobara et al., 2006; Sorensen et al., 2006; Stewart et al., 2011; Gundale et al., 2012; Lett and Michelsen, 2014). Such marked increases in snowmelt and water supply to soil systems typical of the Arctic

are not equivalent to the less pronounced growing seasons, in terms of warming and moisture, that characterize high-latitude oceanic environments experienced by ecosystems in the fjords and islands of the Cordillera Darwin, in the subantarctic region of South America (Rozzi et al., 2012).

As expected, P has been shown to stimulate DA in Arctic cyanolichens (Weiss et al., 2005), by constituting the high energy ATP-molecule required for  $N_2$  reduction to  $NH_3$ . In moss carpets of the austral subantarctic region, however, addition of P alone, or in combination with Mo, inhibited DA (Smith, 1984). Because of the strong negative feedback control of DA by available soil N, it has been reported that rates of diazotrophic activity are higher in early stages of postglacial succession, when soil N availability is minimal (Pérez et al., 2014; Arróniz-Crespo et al., 2014). In glacier forelands of the subantarctic region of Chile, the pioneer *Gunnerya magellanica*, in symbiotic association with the *Nostoc* cyanobacteria, was able to fix up to  $30 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  (Pérez et al., 2014, 2016). Similar rates of  $N_2$  fixation up to  $25 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  have been reported for colonizing *Coriaria nuscifolia*, with actinorhizal symbiosis, in glacier forelands in New Zealand (Menge and Hedin, 2009). Likewise, high rates of biological  $N_2$  fixation of up to  $11 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  have been found in biological soil crusts of the subarctic heath vegetation (Sorensen et al., 2006). There is growing evidence that cold terrestrial environments can have considerably high biological N inputs, which can be about one order of magnitude higher than inputs of reactive N via atmospheric N deposition (Vet et al., 2014). Overall, the cryptogamic flora of extratropical biomes contributes importantly to global biological  $N_2$  fixation, which enhances global carbon storage (Elbert et al., 2012).

In glacier forelands of the Strait of Magellan, C, N, and P start to accumulate in soils after the establishment of  $N_2$ -fixing organisms (Pérez et al., 2014). Rates of DA by free-living and epiphyllous diazotrophs in bryophytes tend to increase as well (Pérez et al., 2014), suggesting that nutrient limitation of DA might change during ecosystem development. In this study, we seek to document these changes across a postglacial chronosequence of vegetation and soil development in Darwin's Cordillera, southern Chile, using field experiments to help us understand nutrient limitation of  $N_2$  fixation by diazotrophs as free-living, symbiotic, and epiphylls on bryophytes. We evaluated the following main hypotheses:

1. In the earliest successional site (younger soil and vegetation), where nutrient limitation is highest and soil nutrient contents are the lowest, the response of the *Nostoc-Gunnerya* symbiosis to C, Mo, and P addition will

be greater than diazotrophs in later successional phases. On the other hand, in the earliest successional site, N addition will have less effect on DA (i.e., less degree of inhibition of DA) because soils have the lowest N availability compared to later successional sites.

2. Additionally, diazotrophs associated with plants either symbiotically or as epiphylls on bryophytes with high Mo and P content in their tissues will present the highest rates of DA and a lower response to nutrient addition.

3. Heterotrophic diazotrophs will respond more strongly to carbon addition than symbiotic or epiphyllous diazotrophs.

4. Nutrient limitation will vary seasonally, with more pronounced limitation during the colder autumn season than in the warmer spring.

## STUDY SITES AND METHODS

### Study Sites

The Reina Isabel II Glacier belongs to the Cordillera Darwin ice field (54°41'S, 69°23'W; Fig. 1). In glacial forelands of Glacier Reina Isabel II, we identified for sampling three successional stages characteristic of local soil and vegetation development (Fig. 1). The youngest stage (the early successional site, Fig. 1) was dated ca. 6 yr B.P. (Arróniz-Crespo et al., 2014) and was located right in front of the proglacial lake. We selected two other study sites, located approximately 900 m and 1350 m distant from the proglacial lake, respectively (Fig. 1). At these two sites, tree cores were taken from the four individual trees of *Nothofagus magellanica*, which were the tallest and had the largest stem diameters. Based on

tree-ring counts and cross-dating, the second study site was considered midsuccessional, because the largest tree was 57 years old. The third site was assigned to the late (or more advanced) stage of vegetation and soil development, because the largest tree was 150 years old. Meteorological records (years 1968–2014) from the nearest station located ~100 km southwest of the study site indicated a mean annual precipitation of 486 mm and a mean annual temperature of 6 °C (Dirección Meteorológica de Chile, <http://164.77.222.61/climatologia>). During the sampling period (two subsequent springs), HOBO-data loggers located at the surface of the top soil recorded the following temperatures: in the early successional site, a minimum of 1.2 °C and a maximum of 14.1 °C; in the midsuccessional site, a minimum of 2.3 °C and maximum of 15.5 °C; and in the late successional site, a minimum of 1.9 °C and a maximum of 10.9 °C. During the sampling period of autumn 2015, the early successional site recorded a minimum of –3.0 °C and a maximum of 6.5 °C; the midsuccessional site, a minimum of –2.1 °C and a maximum of 12.8 °C; and the late successional site, a minimum of –2.1 °C and a maximum of 7.1 °C. In spring (November 2014), mean air humidity recorded during the sampling period ranged from 91% in the early to 96% in the late successional site. During autumn sampling (April 2015), mean air humidity ranged from 84% in the midsuccessional site to 95% in the most recently deglaciated site. These data document the maritime influence on the climate of the study area, suggesting that climatic variability is low within seasons.

The glacial sediments in the early successional site were covered by a dense carpet of the stoloniferous, summer green, perennial herb *Gunnera magellanica*,

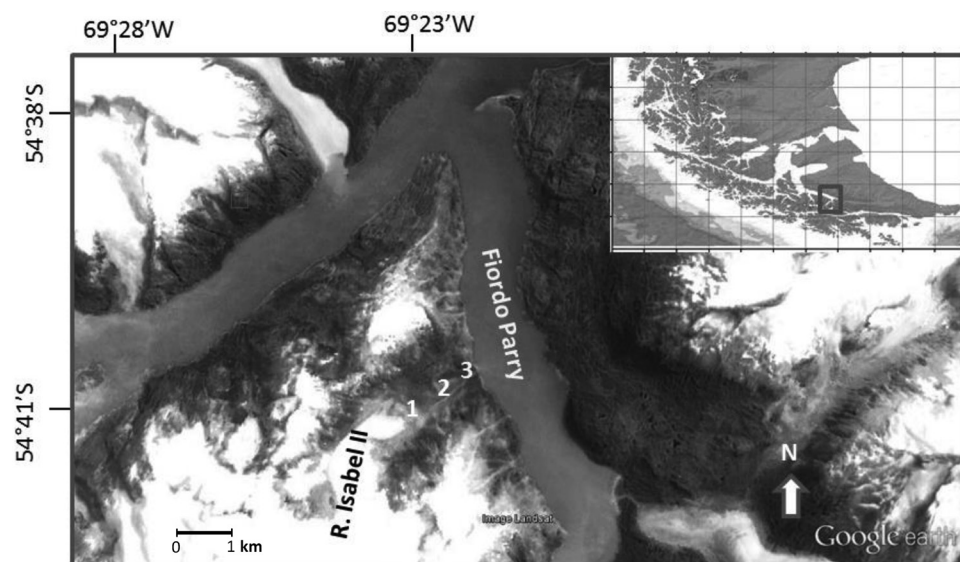


FIGURE 1. Study area in the Cordillera Darwin ice field in Tierra del Fuego, Chile (small rectangle in the map on the right) and successional sites (1–3) in the glacier foreland chronosequence of Reina Isabel II glacier. 1: Early; 2: Mid-; and 3: Late successional sites (see text for successional ages).

which presents a symbiotic association with the cyanobacteria *Nostoc*. A soil crust composed of about 10 species of bryophytes, including the mosses *Andreaea patagonica* and *Sistichium* spp., is present underneath the herbaceous layer. The midsuccessional site is best described as heath vegetation conformed by the low shrubs *Empetrum rubrum* and *Gaultheria mucronata*. Sparse trees of *Nothofagus betuloides* and *Nothofagus antarctica* are also present at this site. The soil surface is covered by a dense cryptogamic layer, dominated by the lichen *Pseudocyphellaria freycinetii*, with cyanobacteria conforming internal papillose cephalodia, and mosses such as *Racomitrium* sp., *Ditrichium* sp., and *Dricranoloma* sp., among the 23 bryophyte species identified that have epiphyllous associations with cyanobacteria (Arróniz-Crespo et al., 2014). The late successional site is a short stature woodland of *N. betuloides*, without shrubs, but with a highly diverse and dense bryophyte cover on the forest floor, including 25 species, most of them liverworts, such *Gakstroemia magellanica* and *Lepidogyna* sp., both of which present epiphyllous associations with  $N_2$ -fixing cyanobacteria (Troncoso et al., 2013).

## Samples of $N_2$ -Fixing Communities and Element Addition Experiments

Random samples of fine-litter layer (recently fallen leaf-litter; O1 soil horizon) containing free-living diazotrophs and samples of tissues of the corresponding dominant herb, and lichen species that presented symbiotic associations with cyanobacteria, and of bryophytes with epiphyllous associations, were taken from each successional site, on two consecutive springs (2013 and 2014) and in autumn 2015. The following symbiotic associations were sampled in the spring 2013 and 2014: Underground stems of the pioneer *Gunnera magellanica* from early succession, specimens of the cyanolichen *Pseudocyphellaria freycinetii* from the midsuccessional site, and biomass samples of the diverse bryophyte carpet with epiphyllous associations covering the forest floor from the late successional site. Considering that the presence of bryophyte-cyanobacteria associations in the study area has been recently documented (Arróniz-Crespo et al., 2014), and for comparative purposes, in autumn 2015, we sampled the bryophyte carpets of each of the three successional sites. Litter samples from the early successional site consisted of senescent foliage and stems of the herb *G. magellanica*, with *Nostoc* symbionts restricted to underground shoots (spring 2013 and 2014). In the midsuccessional heath community and the late-successional woodland, the O1 horizon consisted mostly of recently fallen leaves and small twigs of *Nothofagus betuloides* (sampled during the three consecutive years).

For each study site and stage of succession, samples of  $N_2$ -fixing substrates were homogenized as much as possible by hand. A volume of fresh sample, as similar as possible among all replicates, was placed inside 130 mL glass jars. The final amounts of sample used in the incubations, referred to dry weight, were as follows: 1.7–3 g of *Gunnera-Nostoc* symbiosis (early successional site), 1–1.7 g of the lichen *P. freycinetii* (midsuccessional site), and 0.8–1.3 g of the bryophyte carpet (late-successional site). Regarding senescent material, 1.8–2.4 g of *Gunnera* litter from the early-successional site, and 1.8–3.5 g of fine leaf litter material from the mid- and late-successional sites were used. Moreover, in April 2015, samples of the bryophyte carpet from each study site were incubated, using the following substrate dry weights:  $11 \pm 2.2$  g from the early successional site (including glacial sediment adhered to the biological crusts),  $2.08 \pm 0.7$  g from the midsuccessional site and  $1.1 \pm 0.3$  g from the late-successional site. Nutrients were experimentally added to the incubation jar by injecting a 10 mL solution per jar, using concentration ranges that were expected to have effects on DA according to the literature (Reed et al., 2007; Barron et al., 2008; Reed et al., 2013). We used the following chemical solutions and element concentrations in nutrient addition experiments: 2 mg Mo  $kg^{-1}$  applied as  $Na_2MoO_4$ , 3 g N  $kg^{-1}$  supplied as  $(NH_4)_2SO_4$ , 1 g P  $kg^{-1}$  added as  $KH_2PO_4$ , and finally 6.3 g C  $kg^{-1}$  in the form of glucose. Solutions were added to the jars containing the specific  $N_2$  fixer community type. Experimental treatments were as follows: (1) control samples at field moisture levels; (2) control for solution additions, which consisted of deionized water added only (DIW); (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; and (8) addition of C + Mo + P. Treatments 7 and 8 were applied at the same final concentrations as the respective single-element treatments. The highest variation in moisture content produced by water addition to litter samples was from 55% to 82% and from 86% to 92% in the samples of the bryophyte carpet from late-successional site in November 2014. During each field campaign, 7 replicates (6 with acetylene + 1 without acetylene, see below) per treatment (8 treatments overall) were set up for a total of 56 samples per  $N_2$  fixer community type (free living, symbiotic, and epiphylls), making a total of 112 samples incubated per successional site. This procedure and the subsequent acetylene reduction assays were completed during in situ experiments conducted for three consecutive years during the austral spring (November) 2013 and 2014 and autumn (April) 2015. We assumed that the samples collected each season reflected the current litter decomposition status, soil

weathering, and climatic conditions of the respective season, which ultimately determine the response of the sample, such as the contents of limiting elements for DA. Moreover, we assumed that climatic conditions while performing the field assays should reflect the mean climatic conditions of the corresponding season.

## Acetylene Reductions Assays

Diazotrophic activity was estimated in field incubations using the acetylene reduction assay (Myrold et al., 1999). After nutrient application, samples were allowed to equilibrate overnight. On the next day, acetylene was applied (see below) and the samples were incubated in the field for up to 2 days for litter and up to 8 hours for symbiotic and epiphyllous associated species. Incubations were carried out inside hermetically closed glass jars containing a mixture of air and acetylene at 10% v/v. For each  $N_2$  fixer community type, and each successional site, one sample per treatment was incubated without acetylene to serve as a control for basal ethylene production. Three gas samples were taken periodically from each jar during the incubation period and injected into 4 mL Becton Dickinson Vacutainers for subsequent lab analyses. 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, 1 m\*1/4"ss, 80/100 mesh; Bellefonte, Pennsylvania, U.S.A.) and a flame ionization detector. Ethylene concentration was assessed from a calibration curve by diluting 100 ppm ethylene standard from Scotty analysis gases (Bellefonte, Pennsylvania, U.S.A.). Acetylene reduction activity (ARA) was estimated from the slope of the linear fit of the ethylene production plot during the period of incubation in the jar headspace and referred to dry weight of the sample. Ethylene production in equivalent samples incubated without acetylene for each treatment was subtracted from the production in samples with acetylene. The mean ethylene concentration in the control samples at the field moisture level of free-living diazotrophs varied from 0 to 0.41 nmol ethylene  $g^{-1}$  DW  $h^{-1}$  and in symbiotic and epiphyllous diazotrophs from 0 to 19.24 nmol ethylene  $g^{-1}$  DW  $h^{-1}$ .

## Biomass Estimate

In May 2015, six 15 × 15 cm polyvinyl chloride (PVC) squares were randomly placed in each study site, and the entire aboveground biomass deposited on the ground inside the square was picked up using a small shovel and placed inside zip-lock bags. In the laboratory, each sample was separated by  $N_2$  fixer community type

(*Gunnera magellanica*, bryophyte carpet, *Pseudocyphellaria freycinetii*, and litter), oven dried at 70°C for at least two days, and then weighted. In this way, in addition to the biomass of each  $N_2$  fixer community type we also obtained estimates of DA on an area basis, which were compared to rates reported for other biomes (see Table 4 later in report).

## Chemical Analyses of Plants and Soils

For each successional site, in November 2013, we collected six random samples of each of the following for chemical analyses: surface soil (Ah: 0–10 cm), fine litter (Ol), and plant samples of symbiotic (*Gunnera magellanica*, *Pseudocyphellaria freycinetii*) and epiphyllous (bryophyte carpet) associations used for acetylene reduction assay. At the laboratory, fresh soil samples were sieved through a 2 mm mesh size. 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). Inorganic available N was extracted in a 0.021 M  $KAl(SO_4)_2$  solution (1:4). Ammonium and nitrate were determined by means of fractionated steam distillation (Pérez et al., 2014). Total concentrations of elements were determined from sieved, dry and mechanically ground samples. Total carbon (C) and N in plants and soils were determined by flash combustion in a Carlo Erba NA 2500 element analyzer (Milan, Italy). Total phosphorus (P) of soil and plant material was extracted with a concentrated sulfuric acid/water peroxide solution in a Hach Digesdahl digester (Loveland, Colorado, U.S.A.) and determined by colorimetry with the molybdenum-blue method. Analyses were performed at the Biogeochemistry Laboratory, Pontificia Universidad Católica de Chile, Santiago. Total concentrations of Mo in soil and plant material were determined in a graphite furnace AAS at the Geobotany Laboratory, Universität Trier, Germany, after extraction with a nitric acid/water peroxide solution.

## Statistical Analyses

After testing for homogeneity of variances, either one-way ANOVAs with a posteriori Tukey or Kruskal-Wallis with multiple comparison tests were applied in order to evaluate significant differences in chemical properties of soils and plants among the three successional sites in the postglacial chronosequence. For each study site, data of ARA from the springs of 2013 and 2014 were averaged to obtain a mean value for spring. Data were box-cox transformed (Chen and Lockhart, 1997) owing to the high frequency of null values. Nutrient limitation of DA for each  $N_2$  fixer community type

in each successional site, and for each season (spring: an average from spring 2013 and 2014, and autumn: April 2015) was evaluated using one-way ANOVA (factor = eight element addition treatments) and a posteriori Tukey's tests. Differences were considered statistically significant at  $P < 0.05$ . The software used was Statistica 7. As all elements were applied in solution, only comparisons among treatments with the proper DIW control were considered for the interpretation of nutrient limitation.

## RESULTS

### Chemical Parameters of Soils and Plants

Surface soils from the early successional site (ES) presented the smallest contents of total C, N, and available P and the lowest C/N ratios. Moreover, soils of the ES site presented significantly lower total P than the midsuccessional site (MS), and lower available N than the late-successional (LS) site (Table 1). Surface soils presented similar Mo content in the three successional stages (Table 1). The senescent material from pioneer *G. magellanica*, which was used in acetylene reduction assays, presented the highest contents of Mo and P (Table 2). Living tissues of *G. magellanica* in ES presented the highest content of P and Mo, significantly higher ARA and total N, and lower C/N ratios than samples of bryophyte carpets from LS (Table 3). The cyanolichen *P. freycinetii* of MS presented similar ARA, total C, N, and C/N ratio than the corresponding  $N_2$  fixer communities of both ES and LS (Table 3).

### Nutrient Addition Experiments

During spring, we found no statistically significant response to nutrient addition by senescent material of *G.*

*magellanica* from ES (Fig. 2), or by the free-living diazotrophs in the litter layer of MS (Fig. 2). However, the addition of N and P significantly inhibited ARA in the litter layer of LS (Fig. 2). Control samples at the field moisture levels of *G. magellanica* from ES (Fig. 3, Table 3) exhibited the highest rates of DA during spring incubations (615 nmol ethylene  $g^{-1} h^{-1}$ , Table 3). This value was one order of magnitude higher than for the cyanolichen *P. freycinetii* (60 nmol ethylene  $g^{-1} h^{-1}$ , Table 3) from MS and about 500 times higher than for bryophyte carpets covering the forest floor of LS (Fig. 3, Table 3). Addition of N as ammonium sulfate significantly inhibited ARA in *P. freycinetii* from MS, but no stimulation or inhibition of ARA was observed by the addition of other nutrients (Fig. 3).

During autumn, there was no significantly response to nutrient additions by free-living diazotrophs in the litter of MS (Fig. 4), but while the water addition treatment enhanced ARA, the addition of N inhibited it in LS (Fig. 4). Regarding the bryophyte layer in ES, addition of N significantly inhibited ARA (Fig. 5), but had no effect on substrates from MS or LS. Mo and C added separately significantly enhanced ARA up to five times in the bryophyte carpet of LS (Fig. 5).

During autumn, addition of C + P + Mo simultaneously significantly increased ARA up to 30 fold in the symbiotic pioneer *G. magellanica* (Fig. 6).

## DISCUSSION

### Chemical Parameters of Soils and Plants

Despite that, soils of ES had the lowest level of available P and no difference in concentrations of total Mo; living samples the pioneer *G. magellanica*, symbiotic with *Nostoc*, presented the highest content of these two

TABLE 1

Mean total content ( $\pm 1$  SE) of elements potentially limiting diazotrophic activity, biologically available  $N_a$  and  $P_a$ , and C/N ratios in surface soils of three successional sites in the postglacial chronosequence of Reina Isabel II glacier, Cordillera Darwin, Tierra del Fuego, Chile. Different letters indicate significant differences among successional sites according to Tukey's or multiple comparisons test ( $n = 6$  replicates in each case). ES = early successional site, MS = mid-successional site, and LS = late successional site (successional ages are given in the text).

	ES	MS	LS
C (%)	0.27 $\pm$ 0.12a	31.59 $\pm$ 7.31b	28.9 $\pm$ 6.73b
N (%)	0.017 $\pm$ 0.01a	1.03 $\pm$ 0.22b	0.98 $\pm$ 0.20b
P (mg $kg^{-1}$ )	0.066 $\pm$ 0.015a	0.269 $\pm$ 0.057b	0.123 $\pm$ 0.02ab
Mo (mg $kg^{-1}$ )	0.298 $\pm$ 0.126a	0.409 $\pm$ 0.061a	0.615 $\pm$ 0.061a
C/N	14.87 $\pm$ 1.03a	29.71 $\pm$ 1.51b	28.91 $\pm$ 1.16b
$P_a$ (mg $kg^{-1}$ )	28.18 $\pm$ 1.61a	187.91 $\pm$ 48.87b	235.1 $\pm$ 39.1b
$N_a$ (mg $kg^{-1}$ )	6.63 $\pm$ 1.1a	36.83 $\pm$ 8.38ab	111.52 $\pm$ 17.89b

TABLE 2

Mean content ( $\pm 1$  SE) of elements potentially limiting diazotrophic activity and C/N ratios in the litter layer of the three successional sites in the postglacial chronosequence of Reina Isabel II glacier, Cordillera Darwin, Tierra del Fuego, Chile. Measurements of acetylene reduction activity (ARA) in control spring samples at field moisture level (nmol ethylene  $\text{g}^{-1} \text{h}^{-1}$ ) are also given. Different letters indicate significant differences among successional sites for each variable according to Tukey's or multiple comparisons test. ( $n = 6$  replicates in each case).

	ES*	MS	LS
C (%)	28.59 $\pm$ 2.68 <sup>a</sup>	54.41 $\pm$ 0.39 <sup>ab</sup>	56.87 $\pm$ 0.18 <sup>b</sup>
N (%)	1.9 $\pm$ 0.2 <sup>b</sup>	1.33 $\pm$ 0.09 <sup>b</sup>	0.81 $\pm$ 0.02 <sup>a</sup>
P (mg $\text{kg}^{-1}$ )	0.480 $\pm$ 0.05 <sup>a</sup>	0.206 $\pm$ 0.021 <sup>b</sup>	0.226 $\pm$ 0.02 <sup>b</sup>
Mo (mg $\text{kg}^{-1}$ )	3.177 $\pm$ 0.391 <sup>a</sup>	0.352 $\pm$ 0.08 <sup>b</sup>	0.223 $\pm$ 0.081 <sup>b</sup>
C/N	15.18 $\pm$ 0.46 <sup>a</sup>	42.00 $\pm$ 3.43 <sup>ab</sup>	70.74 $\pm$ 1.52 <sup>b</sup>
ARA	0.54 $\pm$ 0.22 <sup>a</sup>	0.10 $\pm$ 0.03 <sup>a</sup>	0.03 $\pm$ 0.01 <sup>a</sup>

\*Measurements for senescent individuals of *Gunnera magellanica*. Site codes as in Table 1.

TABLE 3

Mean content ( $\pm 1$  SE) of elements potentially limiting diazotrophic activity and C/N ratios in plant tissues of symbiotic and epiphyllous diazotrophs from different successional sites in the postglacial chronosequence of Reina Isabel II glacier, Cordillera Darwin, Tierra del Fuego, Chile. Measurements of ARA (nmol ethylene  $\text{g}^{-1} \text{h}^{-1}$ ) of control spring samples at field moisture level are also given. Different letters indicate significant differences among sites for each variable according to Tukey's or multiple comparisons test ( $n = 6$  replicates for each plant material tested).

	<i>Gunnera magellanica</i>	<i>Pseudocypbellaria freycinetii</i>	Forest floor bryophytes
C (%)	19.17 $\pm$ 1.8 <sup>a</sup>	49.37 $\pm$ 0.23 <sup>ab</sup>	47.09 $\pm$ 0.4 <sup>b</sup>
N (%)	1.06 $\pm$ 0.08 <sup>a</sup>	1.98 $\pm$ 0.03 <sup>ab</sup>	0.73 $\pm$ 0.04 <sup>b</sup>
P (mg $\text{kg}^{-1}$ )	0.390 $\pm$ 0.072 <sup>a</sup>	0.170 $\pm$ 0.041 <sup>b</sup>	0.249 $\pm$ 0.041 <sup>b</sup>
Mo (mg $\text{kg}^{-1}$ )	1.944 $\pm$ 0.505 <sup>a</sup>	0.226 $\pm$ 0.04 <sup>b</sup>	0.262 $\pm$ 0.116 <sup>b</sup>
C/N	18.01 $\pm$ 0.46 <sup>a</sup>	24.94 $\pm$ 0.43 <sup>ab</sup>	65.18 $\pm$ 3.25 <sup>b</sup>
ARA	615 $\pm$ 110 <sup>a</sup>	60.37 $\pm$ 16.14 <sup>a</sup>	1.29 $\pm$ 0.27 <sup>b</sup>

elements compared to biomass samples of  $\text{N}_2$ -fixing organisms from other successional stages in this postglacial chronosequence. This result strongly suggests that *Nostoc* symbionts actively accumulate these essential nutrients, presumably before the colonization of their host plant. As the pioneer *G. magellanica* has no documented mycorrhizal association, we infer that most P in plant tissues must have been acquired by its cyanobacteria symbiont. Similarly, it has been documented that cyanobacteria are able to accumulate and store P in the form of polyphosphates (Smith, 1984). The ability to accumulate Mo has also been reported for symbiotic diazotrophs using a specialized storage system within the cell (Bellenger et al., 2011). As a consequence, DA by the pioneer *G. magellanica* was high despite the Mo-poor soils where it occurs in ES.

### Nutrient Limitation of Diazotrophic Activity

Our results supported hypothesis 1, as ARA in *G. magellanica* of ES, which is associated with the lowest soil nutrient content, increased about 30 times when the elements C, P, and Mo were added in combination. However, when Mo and C were applied singly to the bryophyte carpet of later successional stages, ARA increased about 4–5 times, despite higher soil nutrient content. Similar positive effects of the addition of P and Mo alone or in combination on ARA of free-living diazotrophs is known to occur in tropical forests (see review in Reed et al., 2013). In the postglacial chronosequence studied, we report that C is also needed to increase symbiotic diazotroph activity when adding P +



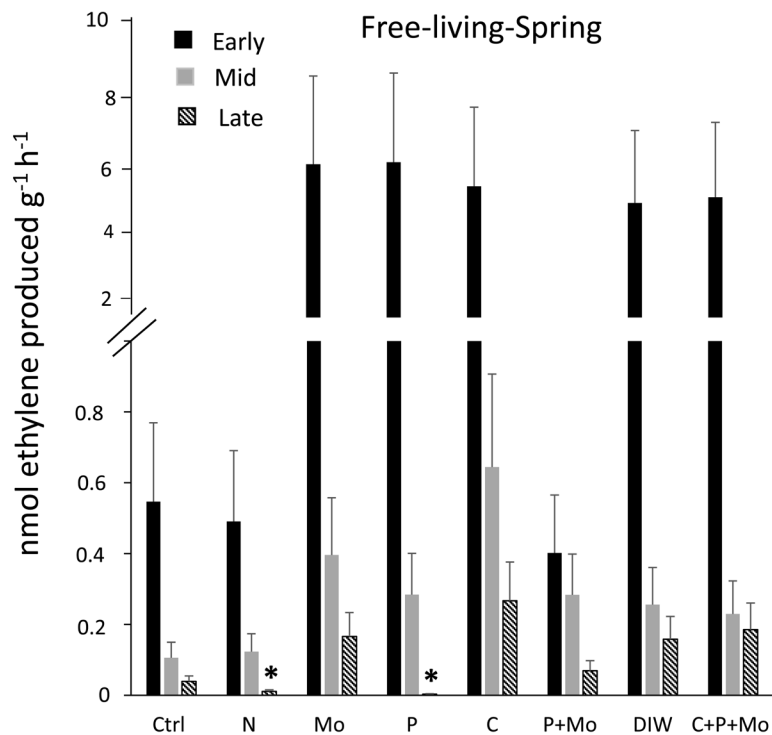


FIGURE 2. Mean acetylene reduction activity ( $\pm 1$  SE) measured during the spring season (2013 and 2014) in senescent tissues of *Gunnera magellanica* containing the symbiotic associations from early successional site and in the fine-litter layer from mid- and late successional sites in the postglacial chronosequence of Reina Isabel II glacier, Cordillera Darwin, Tierra del Fuego, Chile. Nutrient addition experiments evaluated the effects of eight different treatments ( $n = 6$  replicates per treatment). Asterisks indicate significant differences with respect to the DIW control. Ctrl = control samples at the field moisture level, N = nitrogen addition as ammonium sulfate, Mo = molybdenum added alone, P = phosphorus single added alone, C = carbon added as glucose alone, P + Mo = both elements added in combination, DIW = addition of deionized water, C + P + Mo = the three elements added in combination.

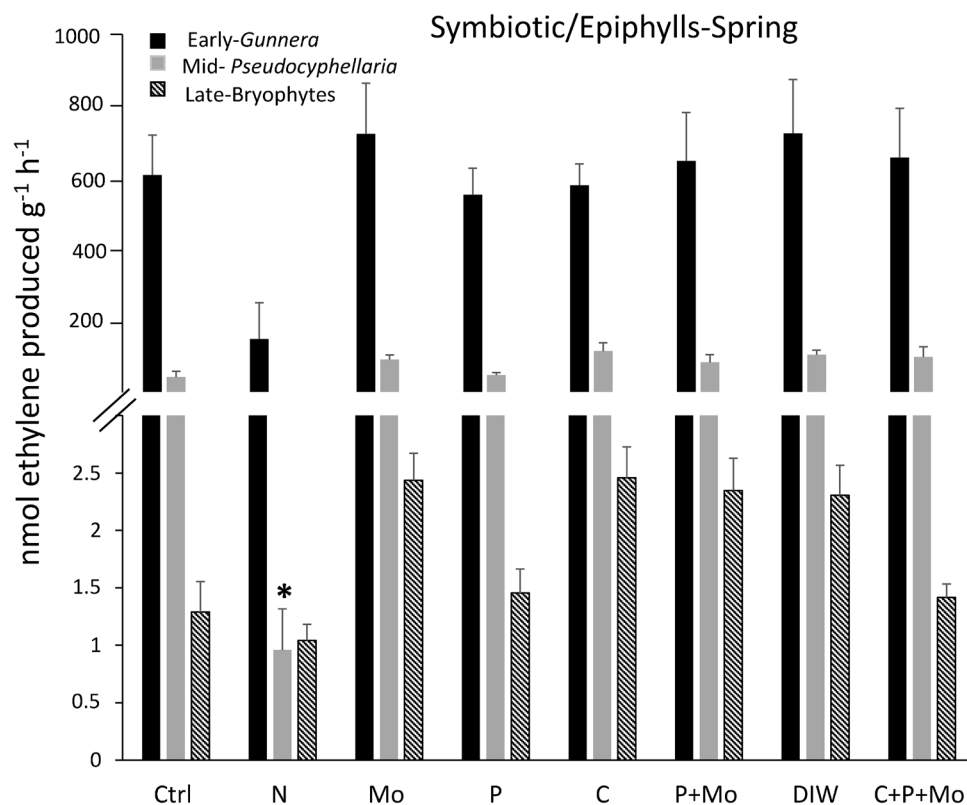


FIGURE 3. Mean acetylene reduction activity ( $\pm 1$  SE) by *Gunnera magellanica* symbionts in early successional site, by the symbionts of the lichen *Pseudocyphellaria freycinetii* in mid-successional site, and by epiphyllous diazotrophs associated with the bryophyte carpet in the late successional site in the postglacial chronosequence of Reina Isabel II in Cordillera Darwin, Tierra del Fuego, Chile. Acetylene reduction activity (ARA) was evaluated during in situ incubations in spring (2013 and 2014), assessing the effects of element additions using eight different experimental treatments. Values are means of  $n = 6$  replicates per treatment. Asterisks indicate significant differences with respect to the DIW control (see text). Legends on the x-axis are the same as in Figure 1.

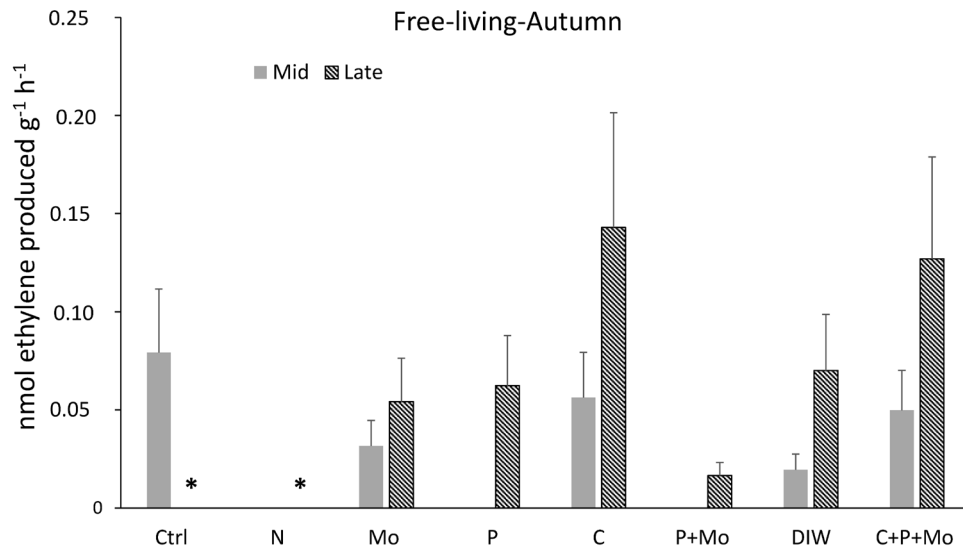


FIGURE 4. Mean ARA ( $\pm 1$  SE) of free-living diazotrophs present in the litter layer of mid- and late successional sites in the postglacial chronosequence of Reina Isabel II glacier, Cordillera Darwin, Tierra del Fuego, Chile. Nutrient addition experiments were conducted in autumn 2015 using eight different treatments ( $n = 6$  replicates per treatment). Asterisks indicate significant differences with respect to the DIW control. Legends on the x-axis are the same as in Figure 1.

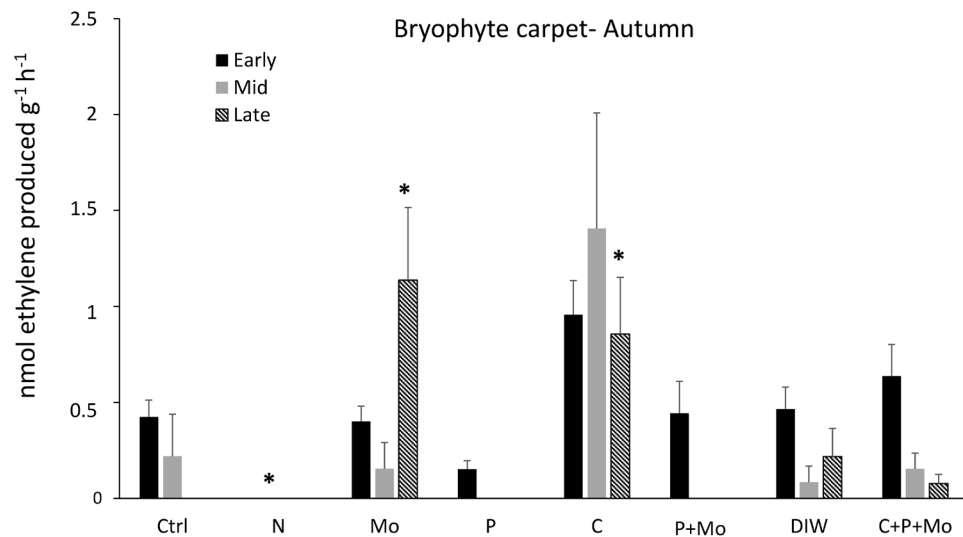
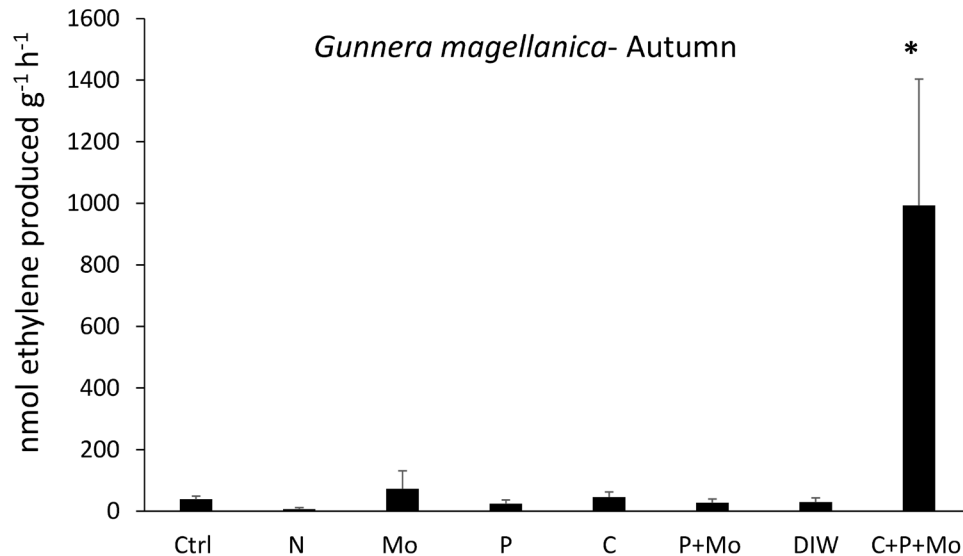


FIGURE 5. Mean ARA ( $\pm 1$  SE) measured in epiphyllous diazotrophs associated with the bryophyte carpets of early, mid-, and late successional sites in the postglacial chronosequence of Reina Isabel II glacier, Cordillera Darwin, Tierra del Fuego, Chile. Field experiments were conducted in autumn 2015 to assess the effects of eight different experimental treatments ( $n = 6$  replicates per treatment). Asterisks indicate significant differences with respect to the DIW control. Legends on the x-axis are the same as in Figure 1.

Mo. It has been reported (Meeks, 2009) that *Nostoc* can shift to a heterotrophic pathway of C fixation when living in symbiosis, taking C from its partner in exchange for available N, a pathway of C fixation that may also occur in the symbiosis of *Nostoc* and *G. magellanica*, as well as in epiphyllous diazotrophs present on the bryophyte carpet in our study sites. Neither in MS nor in

ES did we record an increase in ARA when adding P alone or in combination with Mo. Lack of response of ARA to P addition has also been reported for diazotrophs associated with the moss *Pleurozium shreberi* in boreal forests (Markham, 2009), but a positive response was found in Arctic tundra vegetation (Deslippe et al., 2005). Conversely, the addition of P or Mo alone, or



**FIGURE 6.** Mean ARA ( $\pm 1$  SE) by *Gunnera magellanica* symbionts measured during autumn 2015 in field experiments conducted in early successional sites in the postglacial chronosequence of Reina Isabel II in Cordillera Darwin, Tierra del Fuego, Chile. We evaluated the effects of eight different experimental treatments ( $n = 6$  replicates per treatment). Asterisks indicate significant differences with respect to the DIW control. Legends on the x-axis are the same as in Figure 1.

P plus Mo to subantarctic mosses and their associated cyanobacteria produced an inhibition of ARA (Smith, 1984). Although not statistically significant, a tendency toward inhibition of ARA by P and P + Mo addition was observed in the bryophyte carpet of LS in the present study (Fig. 5). In contrast, increases in ARA after P addition were reported to stimulate rates of  $N_2$  fixation by the cyanolichen *Peltigera* in Arctic ecosystems in Alaska, (Weiss et al., 2005). Taken together, these results suggest that responses depend on differential P requirements for the diverse communities of diazotrophs in high-latitude ecosystems.

When N was added experimentally to symbiotic and epiphyllous  $N_2$  fixers from boreal and Arctic ecosystems, significant reductions in the biomass of  $N_2$ -fixing cryptogamic flora were documented, which strongly reduced  $N_2$  fixation rates at the ecosystem level (Weiss et al., 2005; Gundale et al., 2013). In the postglacial chronosequence studied here, we observed an inhibition of ARA by N addition in the lichen *P. freycinetii* in MS. This inhibition was observed in MS bryophyte carpet during autumn as well.

Our results lend partial support to hypothesis 2, because even though the highest rates of ARA were found in *Gunnera-Nostoc* symbionts, which had the highest Mo and P content in plant tissues, this species also responded significantly and positively to nutrient addition. Accordingly,  $N_2$ -fixing symbionts of *G. magellanica* are able to maintain extraordinarily high rates of ARA, provided

that P and C are supplied in abundance, without showing evidence of Mo toxicity at the concentrations applied in the present experiment. On the other hand, bryophyte carpets characterized by low Mo tissue contents and high C/N ratios (more recalcitrant C) were able to increase ARA after addition of Mo and labile C, corroborating the hypothesis that the highest response should be observed when nutrient contents in plant tissues are the lowest.

Surprisingly, and contrary to our hypothesis 3, free-living diazotrophs did not respond to C addition, either alone or in combination with other elements, regardless of season or stage of postglacial ecosystem development. Moreover, we did not find effects of either Mo and/or P addition on ARA. Recently, an alternative V-nitrogenase enzyme has been recognized in relation to  $N_2$  fixation in cyanolichens from boreal ecosystems (Darnajoux et al., 2014). It is possible that this alternate enzyme complex could be operating in free-living diazotrophs in subantarctic ecosystems as well, thus explaining the lack of response to Mo additions. Higher soil N availability in both seasons in LS, is linked to a reduction of ARA in free-living diazotrophs by N addition, which was not detected in ES and MS, suggesting a pre-adaptation of free-living diazotrophic bacteria to site characteristic soil N. In addition to the trends found for epiphyllous diazotrophs presented above, P addition to the litter layer resulted in significant negative effects on ARA, indicating inhibition of spring activity of free-

living diazotrophs in LS. We suggest that this inhibitory effect of P could result from an excess of P in storage places within the cell. It is possible that  $N_2$  reduction to  $NH_3$ , which produces more available P, might be shut down by a regulatory negative feedback mechanism. We only found a significant effect of water addition during autumn, when this treatment significantly stimulated ARA in the litter of LS; suggesting that water is limiting DA only marginally in these oceanic ecosystems.

Regarding hypothesis 4, the highest rate of DA was recorded in control samples of *G. magellanica* during the milder spring season. A positive response to nutrient addition of *G. magellanica* was found only during the less favorable, colder autumn season. Similarly, DA in the bryophyte carpet of the LS responded positively to Mo and C addition only during autumn. These results suggest a seasonal dynamic of nutrient limitation of DA for all successional stages and biological components involved; however, data are unavailable for seasonal variation on the availability of nutrient contents in soils to confirm this hypothesis. Moreover, we do not discard that preexisting conditions of the samples (i.e., their contents of limiting elements) could have an effect on the nature and degree of element limitation to DA (Belnap, 2001). Field studies in northern temperate (Jean et al., 2013) and subarctic forests (Rousk et al., 2015) have reported a strong seasonal pattern in DA in response to temperature and moisture fluctuations as limiting factors. In the same way, in the present study we report that DA in control samples of *Nostoc-Gunnera* association presented three orders of magnitude higher rates in the spring ( $600 \text{ nmol ethylene g}^{-1}\text{h}^{-1}$ ; Fig. 3) than in the autumn samples ( $<0.6 \text{ nmol ethylene g}^{-1}\text{h}^{-1}$ ; Fig. 6). The same trend was observed in control samples of the bryophyte carpet in the late successional site, where DA reached  $1.29 \text{ ethylene g}^{-1}\text{h}^{-1}$  in the spring (Fig. 3) and  $<0.001 \text{ ethylene g}^{-1}\text{h}^{-1}$  in autumn samples (Fig. 5). This evidence suggests a seasonal fluctuation in DA of symbiotic diazotrophs and epiphyllous associations, which was not so evident in free-living diazotrophs where DA in control samples reached values  $<0.1 \text{ ethylene g}^{-1}\text{h}^{-1}$  in both seasons (Figs. 2 and 4).

## Comparison with Other High-Latitude Ecosystems

Rates of DA reported in the present study (Table 4), referred to the biomass of the corresponding  $N_2$  fixer communities, have similar magnitudes as field and laboratory estimates reported for cryptogamic symbionts or free-living diazotrophs from high-latitude terrestrial biomes. The highest ARA rates have been documented for colonies of cyanobacteria forming mats that cover

the mineral soil in boreal forests reaching up to  $237 \text{ } \mu\text{mol ethylene m}^{-2} \text{ h}^{-1}$  (Table 4). The highest ARA for symbiotic diazotrophs from the subarctic region ( $126 \pm 30 \text{ } \mu\text{mol ethylene m}^{-2} \text{ h}^{-1}$ ) was recorded for legumes in heath vegetation with a low ground cover (Rousk et al., 2015). In the present study, we report the highest rates found for a vascular nonlegume herb species, *G. magellanica*, with values up to  $3796 \pm \text{ } \mu\text{mol ethylene m}^{-2} \text{ h}^{-1}$ . Such high values are probably due to the N-depleted substrates and humid environment colonized by *G. magellanica*, which rapidly reaches a profuse cover on recently exposed glacial till. Moreover, according to the estimated age of MS heath vegetation in this post-glacial chronosequence, this  $N_2$ -fixing species allows the incorporation of new N in top soil by increasing the concentration of N by two orders of magnitude for ca. 60 years after glacier retreat.

## CONCLUSIONS

As in other high latitude biomes, symbiont-related DA plays a major role in N accumulation during post-glacial succession in subantarctic ecosystems. In all high-latitude systems, abiotic factors such as light, temperature, and moisture can limit DA. In the present study, we report that C and Mo and P, when added in combination, can have positive effects on DA in the successional sites, and therefore these elements can be limiting DA as well. Positive responses to element addition were more evident during the less favorable, colder autumn season regardless of the stage of postglacial soil and vegetation development. Experimental N addition in the form of ammonium sulfate had a strong inhibitory effect on DA, and this depression of DA was observed for symbiotic, epiphylls, and free-living diazotrophs. Further research should provide insights on the role of V-nitrogenase enzyme, and the different functional groups in litter and cyanobacterial associates of *P. freycineti*, which are important players in the N cycle in the subantarctic region.

In summary, the predicted increase in the rates of glacier melting in southern South America, with the consequent exposure of new substrates, should enhance the probability of colonization by diazotrophic bacteria, provided that key elements, such as Mo and P and C, are present and reactive N is lacking from such substrates and remains low in atmospheric deposition.

## ACKNOWLEDGMENTS

Work supported by the following grants: Fondecyt 1130353 (Pérez), Fondecyt 1030381 (Aravena), and ICM-MINECON P05-002 and CONICYT PFB-23

TABLE 4

Measurements of diazotrophic activity referred to biomass ( $\mu\text{mol ethylene m}^{-2} \text{ h}^{-1}$ ) for symbionts and epiphylls (in cryptogamic flora) and free-living diazotrophs in high-latitude terrestrial biomes and in the present study.

Study Site (Biome, number of replicates)	Latitude	Condition	DA (symbionts/ epiphylls)	DA (free-living)	References
Cordillera Darwin, Chile (subantarctic, $n = 3$ )	54°41'S	Field	3–51	0.06 – 0.5	This study
Abisko, north Sweden (subarctic; $n = 3$ )	68°19'N	Field	9–25	11–48*	Sorensen et al. (2006)
Abisko, north Sweden (subarctic; $n = 5$ )	68°19'N	Field	4–103	3–5	Rousk et al. (2015)
Imnavait Creek, Alaska (arctic tundra; $n = 4$ )	68°37'N	Laboratory	6–10	0	Hobara et al. (2006)
Devon Island, Canada (High Arctic polar desert; $n = 3$ )	75°33'N	Laboratory	18–58	0–12	Dickson (2000)
Svalbard Archipelago, Norway (High Arctic tundra, coastal; $n = 7$ )	78°5'N	Laboratory	n.a.	10–237*	Liengen and Olsen (1997)
Svalbard Archipelago, Norway (High Arctic moss flora; $n = 4$ )	78°17'N	Field	3–43	n.a	Solheim et al. (2006)
Svalbard Archipelago, Norway (High Arctic tundra; $n = 6$ )	78°37'N	Field	1–28	0.1–10	Zielke et al. (2005)

\*Colonies of cyanobacteria.

to the Institute of Ecology and Biodiversity, Chile. We are also grateful to the Laboratory of Geobotany at the University of Trier, Germany, for molybdenum analysis. Two anonymous reviewers greatly helped to improve the manuscript.

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MS submitted 23 February 2016

MS accepted 28 October 2016