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Research

High nitrogen contribution by *Gunnera magellanica* and nitrogen transfer by mycorrhizas drive an extraordinarily fast primary succession in sub-Antarctic Chile

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Summary

- Chronosequences at the forefront of retreating glaciers provide information about colonization rates of bare surfaces. In the northern hemisphere, forest development can take centuries, with rates often limited by low nutrient availability. By contrast, in front of the retreating Pia Glacier (Tierra del Fuego, Chile), a *Nothofagus* forest is in place after only 34 yr of development, while total soil nitrogen (N) increased from near zero to 1.5%, suggesting a strong input of this nutrient.
- We measured N-fixation rates, carbon fluxes, leaf N and phosphorus contents and leaf $\delta^{15}N$ in the dominant plants, including the herb *Gunnera magellanica*, which is endosymbiotically associated with a cyanobacterium, in order to investigate the role of N-fixing and mycorrhizal symbionts in N-budgets during successional transition.
- *G. magellanica* presented some of the highest nitrogenase activities yet reported (potential maximal contribution of 300 kg N ha^{-1} yr^{-1}). Foliar $\delta^{15}N$ results support the framework of a highly efficient N-uptake and transfer system based on mycorrhizas, with *c.* 80% of N taken up by the mycorrhizas potentially transferred to the host plant.
- Our results suggest the symbiosis of *G. magellanica* with cyanobacteria, and trees and shrubs with mycorrhizas, to be the key processes driving this rapid succession.

Introduction

Recently exposed soils after glacier retreat are nutrient-depleted habitats (Yoshitake *et al.*, 2007; Göransson *et al.*, 2011; Schulz *et al.*, 2013). Ecological succession is therefore generally constrained (Tilman, 1990), with nitrogen (N) and phosphorus (P) availability as the most common limiting factors (Vitousek *et al.*, 1989, 2010; Vitousek & Howarth, 1991; Yoshitake *et al.*, 2007; Batterman *et al.*, 2013; Schmidt *et al.*, 2016; Castle *et al.*, 2017). N limitation often occurs in early stages of colonization and is progressively mitigated by the increasing quantity and availability of N in the system coming from deposition and biological processes (Vitousek & Farrington, 1997). Atmospheric deposition is small for most extensive glacier forelands with the exception of the Himalayas (i.e. < 1 kg N ha^{-1} yr^{-1}; Dentener *et al.*, 2006) and the primary sources of N in these areas are N release from the bedrock (Houlton *et al.*, 2018) or biological N-fixation (LeBauer & Treseder, 2008). In the latter case free-living or symbiotic cyanobacteria are classic diazotrophic organisms during early successional stages (Walker *et al.*, 2003; Schmidt *et al.*, 2008; Menge & Hedin, 2009; Raggio *et al.*, 2012; Arróniz-Crespo *et al.*, 2014). Later in the succession, vascular plant colonization and tree establishment can introduce endosymbiotic α-proteobacteria (i.e. *Rhizobium*) or actinobacteria (i.e. *Frankia* with *Alnus*) as more important N fixers (Lawrence *et al.*, 1967; Chapin *et al.*, 1994; Kohls *et al.*, 2003). Surprisingly, the N-fixation by symbiotic associations with vascular plants during primary succession is seldom measured and is therefore possibly underestimated in high-latitude regions.

N availability directly impacts primary productivity which, in turn, rests on photosynthetic activity. Photosynthetic organisms along the succession in glacier forelands are of paramount importance in exponentially increasing the soil organic fraction. Microbial and cryptogamic communities certainly help to ameliorate the initial harsh conditions of barren exposed substrates, but their impact on C stock is small in comparison with the contribution by vascular species (Crocker & Major, 1955; Matthews, 1992; Chapin *et al.*, 1994). However, photosynthetic rates at glacier forelands have been mostly evaluated on cryptogams (Uchida *et al.*, 2006; Yoshitake *et al.*, 2010; De los Ríos *et al.*, 2011;

Raggio *et al.*, 2012), and integrated approaches that also include vascular species are scarce (Muraoka *et al.*, 2002). Thus, improving our knowledge of photosynthesis during primary succession is essential, not only to accurately characterize the major sources of organic C at each successional stage, but also to obtain some level of understanding of possible limitations to C-fixation.

Plant colonization of newly exposed soils in glacier forelands may be rapid (Sattin *et al.*, 2009; Knelman *et al.*, 2012) but forest development, if climate allows tree growth, takes several decades or even hundreds of years (e.g. northern hemisphere: Chapin *et al.*, 1994; Alexander & Burt, 1996; southern hemisphere, Chile: Pérez *et al.*, 2014). However, this understanding has been recently challenged by the rapid succession to a forest that occurs in front of the receding Pia Glacier in Tierra del Fuego, Chile (Sancho *et al.*, 2011). Despite the lack of abiotic N inputs in this region (< 0.7 kg N ha^{-1} yr^{-1}; Dentener *et al.*, 2006), total soil N levels increase from below the detection limit at initial successional stages to 1.5% (DW) after 34 yr (Arróniz-Crespo *et al.*, 2014) when a *Nothofagus*-dominated forest is in place with trees reaching 10 m in height. The rapid soil N enrichment and forest development both suggest that another N source, possibly biological N fixation (usually reported to be a crucial N source in glacier forelands; Chapin *et al.*, 1994), speeds up the succession at Pia Glacier. Free-living and symbiotic diazotrophic microorganisms appear early in the succession (Fernández-Martínez *et al.*, 2016) with cyanobacteria rapidly becoming broadly distributed as symbionts in lichens and the vascular plant *Gunnera magellanica*, and epiphytic on mosses (Sancho *et al.*, 2011; Raggio *et al.*, 2012; Arróniz-Crespo *et al.*, 2014). *G. magellanica* is a native N-fixing herb that is common on Fuegian glacier forelands (Henríquez & Lusk, 2005; Troncoso *et al.*, 2013; Pérez *et al.*, 2014) and, at Pia Glacier, it rapidly dominates the herbaceous layer along the succession and is even present under *Nothofagus* spp. forest canopy (Sancho *et al.*, 2011). *Gunnera* is the only angiosperm taxon known to establish a symbiotic relationship with a cyanobacterium (*Nostoc*) and the unique nature of the endosymbiosis (Osborne & Bergman, 2009) has attracted scientific attention for decades (Silvester & Smith, 1969; Silvester & McNamara, 1976; Bergman, 2002; Bergman & Osborne, 2002). However, *Gunnera* has been surprisingly neglected when studying the role of N-fixers in ecosystem succession, as well as in estimates of biological N-fixation (Cleveland *et al.*, 1999; Vitousek *et al.*, 2013). Thus, although recent studies suggest that the N-fixing capacity of this species may be high (Pérez *et al.*, 2017), more effort is still required to better understand the ecological relevance of *G. magellanica* for biochemical cycles and ecosystem functioning in the sub-Antarctic region of Tierra del Fuego.

Little is known about both the fate of fixed N and the main pathways driving N acquisition by non-N-fixers during primary succession. Mycorrhizal associations are key elements in plant nutrition (Johnson *et al.*, 2016) and have been proposed to be essential during ecological succession (Lambers *et al.*, 2008; Dickie *et al.*, 2013; Johnson *et al.*, 2016). However, many early colonizing plants lack mycorrhizas and this symbiosis has been rarely evaluated in glacier forelands. Mycorrhizas are not only able to increase plant access to recalcitrant and slowly diffusible forms of N but also to take up organic compounds and transfer these to their host (Näsholm *et al.*, 2008, 2013). Whilst their increased surface area and uptake capacities at low external concentrations may alter the average N discrimination ($\delta^{15}N$) of the available N sources, N isotope discrimination is also markedly changed by the transfer of N from mycorrhizas to their host (for a detailed review see Hobbie & Högberg, 2012). In particular, creation of transfer compounds by mycorrhizal fungi leads to retention of ^{15}N-enriched N in the fungus and transfer of ^{15}N-depleted N to the plant symbiont. For this reason, the $\delta^{15}N$ value in plants is an indicator of the occurrence of this process (Kohls *et al.*, 2003; Hobbie *et al.*, 2005). This ^{15}N fractionation during transfer probably occurs in all autotrophic mycorrhizal plants but, normally, it is difficult to directly link plant $\delta^{15}N$ to soil N sources (e.g. the exact $\delta^{15}N$ of the latter is rarely known; Craine *et al.*, 2015). In addition, multiple concomitant factors can also influence natural isotopic ratios and hinder the use of plant or soil isotopic signature to infer N cycling processes (Craine *et al.*, 2009, 2015). However, Pia Glacier foreland is unusual in that initial soil N contents are undetectable (Arróniz-Crespo *et al.*, 2014), atmospheric N deposition rate is one of the lowest in the world (Dentener *et al.*, 2006), bedrock N is at low levels (Holloway & Dahlgren, 2002), and there is an almost immediate disconnection of newly exposed surfaces from the meltwater from the glacier. As a result, almost all N in this glacier foreland probably comes from N-fixation and the site provides the possibility to investigate both sources of N and the role of mycorrhizas in transferring N to non-N-fixing plants.

We measured the *in situ* N-fixing capacity of the flowering plant *G. magellanica* in front of the receding Pia Glacier to determine whether this species could be considered as a substantial source of N in this area. We also measured the C fluxes (photosynthetic activity and respiration) to compare the performance of *G. magellanica* with other codominant species present in the successional sequence. Finally, we determined foliar $\delta^{15}N$ and nutrient contents of dominant species. We hypothesize that: the N-fixing herb *G. magellanica* provides the large quantity of N required to support the extraordinary fast succession at Pia Glacier and has a considerably higher N-fixation rate than N-fixing cryptogamic species in the same area; in addition to their role in obtaining P, mycorrhizas play a key role in N acquisition and, by the transfer process, change the $\delta^{15}N$ content of the host plant; and the photosynthetic activity of *G. magellanica* will be higher relative to other co-occurring non-N-fixing species due to the direct N supply from endosymbiotic cyanobacteria.

Materials and Methods

Site description

The study site lies in the morainic field exposed after the retreat of two branches of Pia Glacier in Pia Bay, on the north side of the Beagle Channel in Tierra del Fuego, Chile (54°46′S, 69°40′W). The climate of the area is dominated by high annual precipitation (1600 mm) with mean annual air temperature of *c.* 4.5°C (Sancho *et al.*, 2011). The deposited

moraine ridges, consisting mainly of gravels, stones and larger rocks composed of crystalline, granitic or high-grade metamorphic materials, were progressively formed by glacier regression. The length of exposure (surface age) of individual moraines was determined by aerial photographs (images from 1990, 2000 and 2006), dendrochronology using cores or cross-sections of *Nothofagus antarctica* (G. Forst.) Oerst. trees, and lichenometry using the fast-growing lichen species *Placopsis perrugrosa* (Nyl.) Nyl. (see Sancho *et al.* (2011) for detailed chronosequence description). Sampling was carried out at five sites with surface ages of 4, 7, 10, 19 and 34 yr. Vegetation colonization and development in the chronosequence was rapid. The initial stages of succession (< 4 yr) were dominated by cryptogamic species, most of which establish cyanobacterial associations (Arróniz-Crespo *et al.*, 2014) but young plants of *G. magellanica* Lam. were also present (Supporting Information Fig. S1). This latter species often dominated with dense patches on all surfaces with ages of 7 yr and older (Fig. 1). Scattered *Nothofagus* seedlings (60 cm high) were already present after 7 yr and, on the oldest site (34 yr), a dense, 10 m high *Nothofagus* forest was present (*N. antarctica* and *N. betuloides* (Mirb.) Oerst.) with the herbaceous layer overwhelmingly dominated by *G. magellanica* (Figs 1b, S1). The seral shrub *Gaultheria mucronata* (L.f.) Hook. & Arn. was present on all sites aged 7 yr and older.

Sampling design

The Pia Glacier foreland was visited on two different occasions during the Austral summers of 2009 and 2015. On the first visit in 2009, plant material was collected from the more common species (Table 1) along the chronosequence in front of the Pia Glacier. Three transects were established 10 m apart running from the youngest surface near the glacier to the oldest close to the shoreline. On each transect, five sites were marked with ages 4, 7, 10, 19 and 34 yr. At each of these sites, mature and sun-exposed leaves were randomly collected from one or two plants of *G. magellanica*, *G. mucronata*, *N. betuloides* and *N. antarctica*, if present, until a total of six samples of each plant species had been obtained (Table 1). Leaf samples were placed in paper bags, immediately dried and then couriered to Waikato University (Hamilton, New Zealand) for element determination (see Green *et al.* (2017) for further description). Soil samples (upper 10 cm, five samples at randomly chosen locations at each surface age) were collected using sterile trowels, placed in sterile Whirl-Pak® (Nasco, Fort Atkinson, WI, USA). They were then frozen immediately and transported frozen to Universidad Complutense, Madrid (Spain), for subsequent laboratory analysis.

On a second visit in 2015, we performed *in situ* measurements of the C fluxes and N-fixation. To do this, we collected complete *G. magellanica* plants from two moraine areas parallel to the

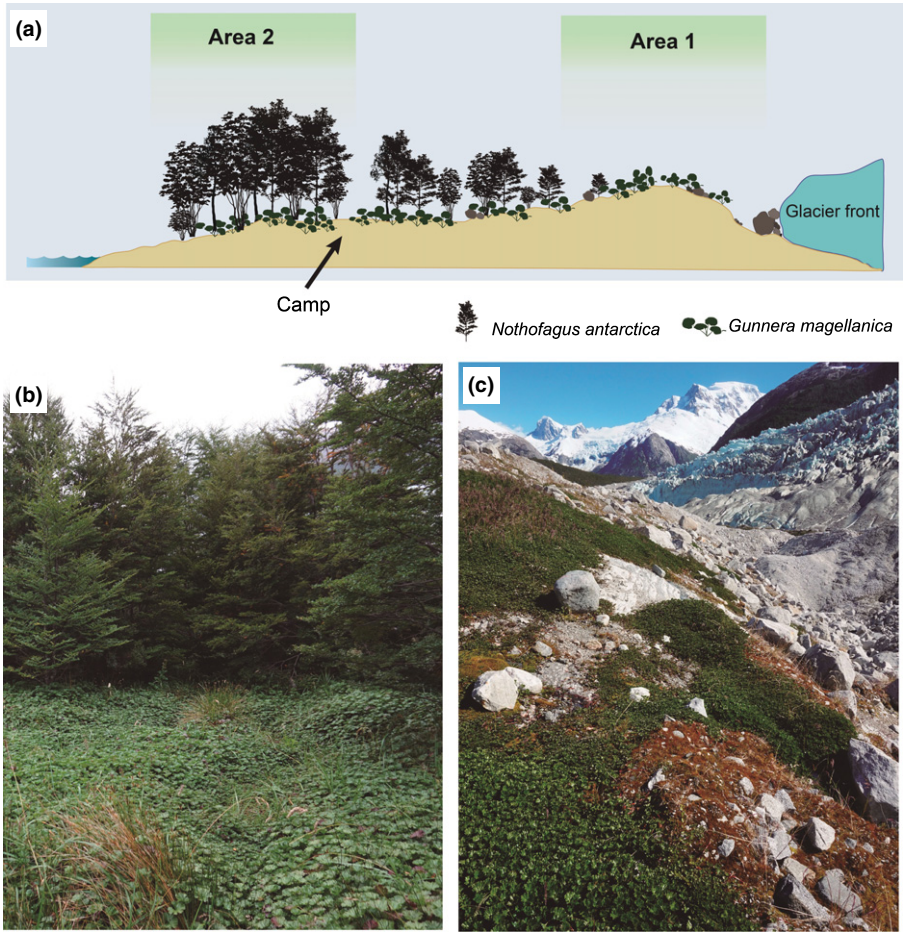

Fig. 1 (a) Schematic description of the glacier foreland in front of Pia Glacier (Tierra del Fuego, Chile). Areas 1 and 2 are the sampling locations (moraine and forest areas, respectively) and the arrow indicates the location of the field camp. (b) *Nothofagus* spp. forest (area 2) with dense cover of *Gunnera magellanica* at the herbaceous layer. (c) Pia Glacier front (area 1) and the initial colonization stages of *G. magellanica*.

Table 1 Plant species selected for leaf element and isotopic (^{15}N) content, indicating the life-form and type of mycorrhizal association, and presence in the chronosequence.

Species	Form	Mycorrhizal type	Presence (site age in years)				
			4	7	10	19	34
Gunnera magellanica	Herb	VAM	+	+	+	+	+
Gaultheria mucronata	Shrub	Eric.		+	+	+	+
Nothofagus antarctica	Tree (deciduous)	Ecto.			+	+	+
Nothofagus betuloides	Tree (evergreen)	Ecto.		+	+	+	+

Mycorrhizal types were obtained from Fontenla *et al*. (2001) and Becerra & Zak (2011). Species distribution along the chronosquence and details of site age determination are provided in Arróniz-Crespo *et al*. (2014).
Ecto., ectomycorrhiza; Eric., ericoid mycorrhiza; VAM, vesicular–arbuscular mycorrhiza.

glacier front (Fig. 1): area 1: immediately close to the glacier front (hereafter moraine area) dominated by cryptogamic species (mainly *Placopsis* spp., *Peltigera patagonica* Räsänen and various mosses) but with initial *G. magellanica* colonization (*c.* 4 yr exposure); and area 2: surface age 40 yr (34 yr in 2009), *c.* 200 m distant from the glacier front, within a *Nothofagus*-dominant forest (hereafter forest area), with *G. magellanica* dominating the herbaceous layer and occasional cryptogams growing on rocks areas in forest clearings (Fig. 1). In both areas, six sampling plots (0.5 × 0.5 m) were randomly selected with at least 10 m separation to ensure independence between plants (*G. magellanica* spreads by stolon production). At each plot, two samples consisting of soil cubes (10 × 10 × 10 cm) containing *G. magellanica* plants (including roots) were collected and transported to our base camp in the forest area (Fig. 1a). One sample was used for photosynthetic measurements and the other for N-fixation analysis (plants were processed differently, see description below). Controls for the possible effects of transplanting on plant photosynthetic activity were included and consisted of similar soil cubes with *G. magellanica* ($n = 5$) naturally growing in the forest close to the base camp (and within measurement range of the equipment). N-fixing lichen species that were locally abundant were also sampled: *Placopsis perrugosa* and *Peltigera patagonica* (only present in the moraine area, used also for N-fixation measurements), and *Stereocaulon alpinum* Laurer ex Funck. Lichen thalli were collected with a similar spatial replication as described for *G. magellanica* and transported to the base camp. The tree species *N. antarctica* was also used for C-fixation analysis with measurements made on exposed leaves of a well-developed tree growing near the base camp.

Carbon fluxes measurements

Carbon fluxes (CO_2-exchange – photosynthesis and respiration) were measured under field conditions using a portable open flow IRGA system (GFS 3000; Walz, Effeltrich, Germany). Measurements were made over four full days for *G. magellanica* and *N. antarctica* samples, and over three full days for lichens. The measuring periods covered a wide range of climatic conditions which are probably representative for the study area during the summer growing season. During C flux measurement, incident photosynthetic active radiation (μmol m^{-2} s^{-1}, PAR), temperature inside the measuring cuvette (°C; Tcuv) and relative humidity (rh) were recorded as these can all influence C fluxes. Leaves of the *N. antarctica* tree ($n = 5$) were measured at the same time as *G. magellanica* ($n = 5$). All samples were measured at intervals through each day. C fluxes of lichens ($n = 5$ for each species) were measured in a similar manner over three complete days. The first day was a dry day, meaning that lichens were inactive, so they were artificially wetted in the evening by spraying with water taken from the glacier. On the remaining 2 d, measurements were made under natural conditions because the lichens had been hydrated by rain.

Leaf nutrient, soil and ^{15}N discrimination analysis

Leaf P analysis was performed by inductively coupled plasma mass spectrometry (ICP/MS) at Waikato University, Hamilton, New Zealand (see Green *et al.* (2017) for details). Leaf N and ^{15}N contents were measured by the Waikato Stable Isotope Unit (WSIU, Waikato University) using their internationally accredited methods. Soil analyses were carried out as follows. Samples were air-dried, sieved (2 mm) and homogenized before analysis. Soil pH was measured for all soil samples with a pH-meter in a 1 : 2.5 mass per volume soil and water suspension. Total soil N and C were measured with an LECO CNS 2000 (LECO Corp., Saint Joseph, MO, USA) auto-analyzer system . Soil nitrate (NO_3^-), nitrite (NO_2^-), Ca^{2+}, Mg^{2+}, Na^+, K^+, PO_4^{3-}, SO_4^{2-} and Cl^- were analyzed using a Metrohm 761 compact ionic chromatograph. Ammonium (NH_4^+) was determined colorimetrically (Arróniz-Crespo *et al.*, 2014). Potential nitrification was determined using the shaken soil slurry method (Belser & Mays, 1980); see Arróniz-Crespo *et al.* (2014) and Green *et al.* (2017) for further description.

Mycorrhizas and ^{15}N discrimination – theory

When mycorrhizas are involved in transferring soil N to their host plants, large changes in plant tissue $\delta^{15}N$ can result. The transfer of ^{15}N-depleted compounds from fungi to plants, coupled with N retention by the fungi, leads to ^{15}N-depleted plants and ^{15}N-enriched fungi and these ^{15}N patterns can provide insight into N partitioning between mycorrhizal fungi and host plants (Hobbie, 2005). The extent of these changes in plant ^{14}N : ^{15}N ratio is influenced by the type of mycorrhizal association formed by plants (Hobbie & Högberg, 2012), with foliar $\delta^{15}N$ decreasing in the order nonmycorrhizal (mean ± SE, 0.9 ± 0.2‰) > arbuscular mycorrhizal (−1.1 ± 0.1‰) > ectomycorrhizal (−2.3 ± 0.2‰) > ericoid mycorrhizal plants (−5.0 ± 0.2‰). In an open system, the proportion of N taken up by mycorrhizal fungi that is then passed on to host plants (termed the transfer ratio; Tr) is determined according to the following equation (Hobbie *et al.*, 2000):

$$\delta^{15}N_{plant} = \delta^{15}N_{available\ nitrogen} - \Delta f \times (1 - T_r) \times f.$$

where Tr is between 0 and 1, f is the proportion of N in the plant that comes from the fungus, and Δf represents the discrimination against ^{15}N during the creation of transfer compounds and is reported to be 8–10‰ (Hobbie & Hobbie, 2008). The rationale is presented in Methods S1.

Biomass and apex density estimates

G. magellanica plants spread by rhizomes so plant density (plants m^{-2}) was determined as the number of rhizome apices (the apex of the rhizome includes the apical bud and also the symbiotic cyanobacterium) in each 10 × 10 cm sample. The projected area of the lichens *P. patagonica*, *P. perrugosa* and *S. alpinum* samples was determined from photographs using ImageJ software version 1.50i (NIH, Bethesda, MD, USA). DW was obtained for *G. magellanica*, *P. patagonica* and *S. alpinum* samples by drying in an oven at 60°C for 72 h, and for *P. perrugosa* (lichen is firmly attached to the rock) by change in weight after burning the samples at 850°C for 2 h.

N fixation analysis

Nitrogenase activity was estimated in parallel to C flux measurements, under field conditions, by using the acetylene reduction assay (ARA, Hardy *et al.*, 1968). *G. magellanica* samples ($n = 5$ for each sampled area) and lichen samples (*P. patagonica*, *S. alpinum* and stones with *P. perrugosa* thalli; $n = 5$ for each species) were enclosed either in 1 liter glass incubation bottles fitted with a rubber septum or wide-mouth glass jars of 125 ml with PTFE-faced silicone septum. Before incubation, samples were prepared by removing attached soil and debris and afterwards kept hydrated by spraying. During incubation, the bottles were placed under *Nothofagus* canopy to avoid warming by direct sun exposure, but with enough light to ensure active photosynthesis (Fig. S2). Temperature, inside and outside the bottles, was continuously monitored with iButton (HomeChip Ltd, Milton Keynes, UK; Fig. S2). For each species, and for each studied area in the case of *G. magellanica*, one extra sample was incubated without acetylene as a control to detect basal ethylene production. Acetylene was produced *in situ* by adding water to calcium carbide inside a modified carbide lamp and stored in 1 liter Tedlar Gas Sampling Bags (Supelco Inc., Bellefonte, PA, USA) until needed (i.e. injected into each jar). Samples were incubated with acetylene at 10% (v/v) and, after 1 h of incubation, a gas sample was extracted using a gas-tight syringe and stored in 5.7 ml Exetainer (Labco Ltd, Lampeter, UK) evacuated gas sampling vials. After each sampling the incubation bottles were aerated by opening the jars to prevent possible long-term effects on nitrogenase activity (Dart & Day, 1971; David & Fay, 1977; Wani *et al.*, 1983). This process was repeated, for the same samples, at 3 h intervals, over a complete 24 h cycle. Exetainer vials were transported to Madrid for ethylene concentration determination by GC (a Varian 3300 gas chromatograph (Varian, Sunnyvale, CA, USA) equipped with a J&W Agilent HP-PLOT Q (Agilent Technologies, Santa Clara, CA, USA) column and a flame ionization detector). After each ARA, the exact incubation volume and ethylene concentration for each sample was determined as described in Methods S2. Nitrogenase activity rates were then calculated from the ethylene concentrations assuming the commonly accepted $C_2H_4 : N_2$ conversion factor of 3 : 1. Rates were then expressed as nmol $C_2H_4\ g^{-1}\ DW\ h^{-1}$ and $\mu g\ N\ g^{-1}\ DW\ d^{-1}$. In addition, nitrogenase activity rates per area unit (nmol $C_2H_4\ cm^{-2}\ h^{-1}$) were calculated using plant density/thallus area data for each species (see description in Methods S2) and potential annual N contribution by the species to the ecosystem was estimated (assuming 100% cover and continuous activity for all species; $kg\ N\ ha^{-1}\ yr^{-1}$).

Statistical analysis

For statistical analyses we performed a repeated-measures approach because samples were not independent of each other as measurements of both N-fixation and C fluxes were performed by repeated sampling of the same individuals at specific intervals. To assess the significant differences of the N-fixation rates of *G. magellanica* plants between different habitats we conducted a two-way permutational multivariate ANOVA (PERMANOVA; Anderson, 2001), based on Euclidean similarity matrix with habitat (two levels: moraine and forest) treated as a fixed factor, and time (seven levels) and replicate (five levels, nested within habitat) treated as random factors. Differences between the N-fixation rates of the three lichen species studied (*P. patagonica*, *P. perrugosa* and *S. alpinum*) were tested by a similar approach, but without considering habitat and with three levels in the species factor. We followed a more complicated approach to test the differences between the photosynthetic activity and respiration of *G. magellanica* plants and *N. antarctica* trees. We assessed the effect of treatment on photosynthetic activity of *G. magellanica*, as well as differences between *G. magellanica* and *N. antarctica*, by conducting a four-way PERMANOVA, with species (two levels) and treatment (three levels: control, transplanted forest and transplanted moraine, nested within species) as fixed factors, and time (12 levels) and replicate (five levels, nested within treatment) treated as random factors. PAR, Tcuv and rh were included as covariates of photosynthesis to account for differences in the measuring conditions between samples. We then followed a similar approach to test the photosynthetic activity and respiration of the two lichens (*P. patagonica* and *P. perrugosa*) used during C flux measurements. *S. alpinum* was initially included but finally discarded from C fluxes measurements because of the low rates measured for this species. For the lichen species we used a two-way PERMANOVA, with species (two levels) as a fixed factor, and time (11 levels) and replicate (five levels, nested within species) treated as random factors, and PAR, Tcuv and rh included as covariates. PERMANOVAs were developed using 9999 permutations (permutation of raw data) with the PERMANOVA+ for Primer v6 statistical package (PRIMER-E, Plymouth Marine Laboratory, Ivybridge, UK). Differences between habitats (in the case of N-fixation), and

between treatment and species (in photosynthesis and respiration assessments) were tested by using pairwise *post hoc* tests in PERMANOVA.

Finally, the influence of morphology on N-fixation rates of *G. magellanica* from both localities was investigated using one-way ANOVA.

Results

N fixation rates

Nitrogenase activity, based on ARA, showed similar, and marked, diel variation for all studied species, with the highest values around midday (12.00–15.00 h) and lowest values before sunrise (04.00–05.00 h; Fig. 2). *G. magellanica* plants from the moraine and forest had the highest mean values for nitrogenase activity on both a DW and an area basis (Fig. 2; Table 2) but the sites differed significantly only on a DW basis ($P = 0.041$; Table S1). The lichen species *P. patagonica* and *P. perrugosa* had similar nitrogenase activities on a DW basis ($P = 0.561$; Table S1), with rates close to those obtained for *G. magellanica* (Table 2). The lichen *S. alpinum* had the lowest nitrogenase activity, on a DW and area basis, and was significantly lower than that of *P. patagonica* and *P. perrugosa* (Tables 2, S1, $P < 0.05$). All lichen species differed from one another when nitrogenase activity was expressed on an area basis ($P < 0.05$; Table S1), with *P. patagonica* having the highest rate (Table 2).

Maximal estimated annual N contribution of *G. magellanica* showed no statistical difference between moraine and forest sites (276 and 327 kg N ha^{-1} yr^{-1}, respectively; $P = 0.996$; Tables 2, S1). For the lichen species, the estimated N contribution was significantly higher for *P. patagonica* (151.8 kg N ha^{-1} yr^{-1}; $P < 0.005$) than the observed value for *P. perrugosa* and *S. alpinum* (48.41 and 27.84 kg N ha^{-1} yr^{-1}, respectively). These annual contributions need to be treated with caution as they are based on daily estimates, continuous activity and 100% cover (Methods S2).

Comparison of carbon fluxes between locations and species

Transplanting had no statistically significant effect on photosynthetic activity of *G. magellanica* plants (Table S2). Net photosynthetic rates (NP, area basis) were significantly higher for *G. magellanica* in the forest ($P = 0.005$; Table S2). Net photosynthetic rates were significantly higher for the herb *G. magellanica* compared to the tree *N. antarctica* ($P = 0.005$; Fig. 3) but NP for both species was saturated at similar light levels (Fig. 3a,b). There were no significant differences in respiration rates between

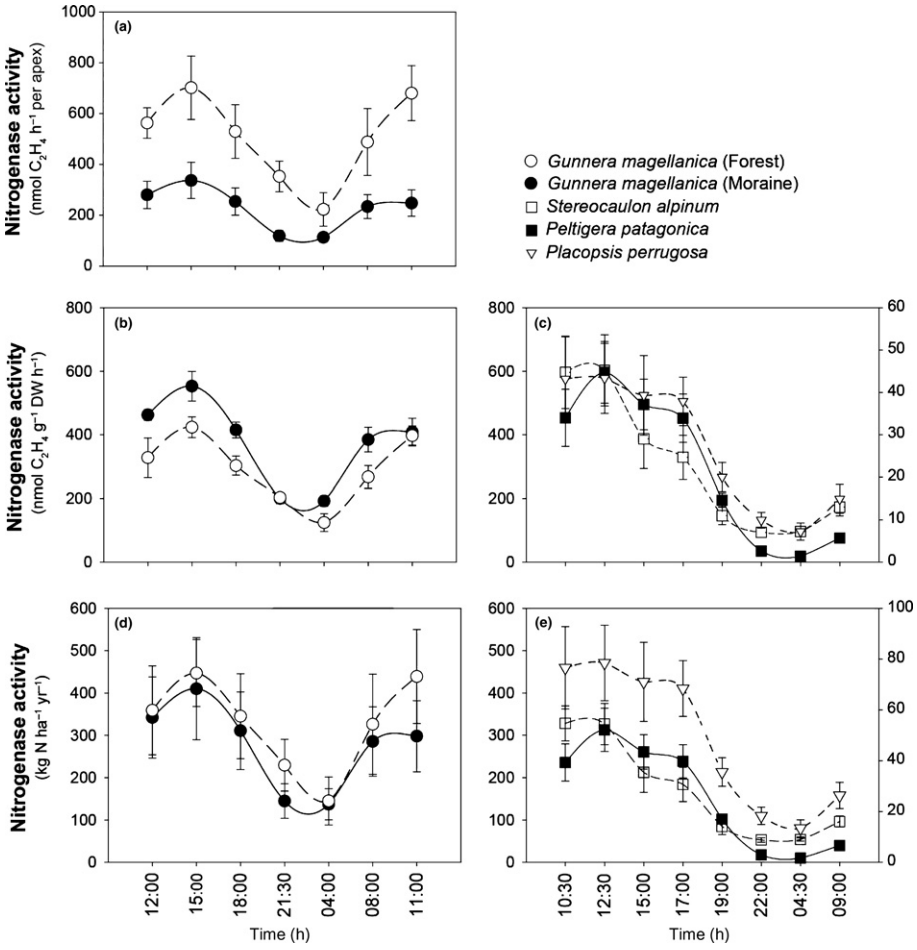

Fig. 2 Nitrogen fixation rates under field natural conditions for the different species considered: *Gunnera magellanica* (a, b, d) and N-fixing lichens (*Stereocaulon alpinum*, *Petigera patagonica*, *Placopsis perrugosa*; c and e). Values are mean ($n = 5$) ± SE for all cases. Note that *S. alpinum* (c, e) and *P. perrugosa* (e) refer to the secondary axis on the right.

G. magellanica from moraine and forest nor between that species and *N. antarctica* (Table S2). The lichens *P. patagonica* and *P. perrugosa* had much lower net photosynthetic rates than both *G. magellanica* and *N. antarctica* but a similar NP response to incident light (Fig. 3c). All species showed similar response of NP to temperature (Fig. 3d–f) with optimum temperatures *c.* 15°C. However, whereas *G. magellanica* had an optimum *c.* 15°C, following a Gaussian curve with the peak very close to the patterns for *N. antarctica*, the optima for the lichen species were less clear.

Leaf N and P contents

G. magellanica leaves consistently had higher N and P content compared to the other analyzed plant species (Table 3; Fig. 4a,b). *G. magellanica* leaves contained three times more N than the shrub species *G. mucronata*, twice that of the evergreen tree *N. betuloides* and 30% higher than the deciduous tree *N. antarctica* (Table 3; Fig. 4a). The seral shrub *G. mucronata* had the lowest N contents, *c.* 1%, whilst *N. antarctica* had N contents *c.* 2.2–3.0% and *N. betuloides* a much lower *c.* 1.5%. The results are very similar for leaf P content with P contents remaining almost constant for each species across all sites (except for *N. betuloides* at early stage, 7 yr; Fig. 4b). However, there were species-specific differences in P content with the same rank order as for N content (*G. mucronata* lowest, then *N. betuloides*, *N. antarctica* and *G. magellanica* highest).

Leaf stable isotope values

Foliar $\delta^{15}N$ results indicated that *G. magellanica* consistently differed from the rest of studied plants along the chronosequence (Fig. 4c). While *G. magellanica* showed $\delta^{15}N$ values close to zero and little change with increasing age of the substrate, *N. antarctica*, *N. betuloides* and *G. mucronata* always had negative $\delta^{15}N$ values and these changed along the chronosequence. For the latter three species, $\delta^{15}N$ was initially low and then rose to be least negative at 10 yr (−2.06, −2.51 and −3.38‰ for *N. antarctica*, *N. betuloides* and *G. mucronata*, respectively). $\delta^{15}N$ then became increasingly negative at older sites with a steady and very similar decline to *c.* −6‰ after 34 yr. *N. antarctica*, *N. betuloides* and *G. mucronata* had very similar $\delta^{15}N$ at the older sites, despite different N contents and mycorrhizal types (Table 1). All species present on surfaces younger than 10 yr had a more negative $\delta^{15}N$, including *G. magellanica* (Fig. 4c). Finally, Tr calculations showed rather consistent values for all studied species (Fig. 4d), with higher differences between species (Tr values between 0.6 and 0.8) in soils younger than 19 yr.

Soil analyses

An impressive feature of the soils along the chronosequence was the very low, often undetectable, levels of key nutrients such as NH_4^+, PO_4^{3-} and Mg^{2+} in the first 10 yr (Table S3). Relatively high levels are only reached at the oldest site when a mature *Nothofagus* forest *c.* 10 m in height has established. Other elements (soil total C, Ca^{2+}, Na^+, SO_4^{2-} and Cl^-) remain almost constant or show a slow rise over the first 19 yr whilst pH shows a slow decline. Potential nitrification rates were below the detection limit at all sites.

Discussion

Our research demonstrates that the Pia Glacier foreland (southern Chile) has characteristics that make it a unique location for investigating and understanding the role of symbioses in underpinning vegetation succession. The early lack of N coupled with rapid forest establishment strongly indicates an important role for biologically fixed N inputs and an efficient N-transfer system. Answering our initial hypothesis, we found that: the perennial herb *G. magellanica* appears to be the most important N-fixing species in the area not only because of its intrinsic high nitrogenase activity rates but also because of its high presence on sites with surface ages of 7 yr and older; the $\delta^{15}N$ values suggest that N is rapidly acquired by shrub and tree vegetation via mycorrhizas; and despite N acquisition by biological N fixation, we found little evidence of enhanced photosynthetic rates in *G. magellanica*. Our results highlight the relevance of *G. magellanica* for ecosystem functioning in Tierra del Fuego and provide a framework that helps to explain the rapid succession in

Table 2 Nitrogen fixation rates (averaged) for evaluated species from both sampling locations obtained from daily cycles of nitrogenase activity measured by acetylene reduction assay.

	Gunnera magellanica			*Peltigera patagonica*	*Pacopsis perrugosa*	*Stereocaulon alpinum*
	Moraine	Forest	Overall	Moraine	Moraine	Forest
Measured rates						
nmol C_2H_4 g^{-1} DW h^{-1}	373.32 (± 50.18)	292.39 (± 39.68)	341.76 (± 15.82)	290.28 (± 6.72)	359.61 (± 34.06)	22.74 (± 2.48)
nmol C_2H_4 cm^{-2} h^{-1}	67.41 (± 9.3)	80.1 (± 10.05)	74. 15 (± 5.7)	37.13 (± 10.6)	11.84 (± 2.4)	6.81 (± 1.65)
Estimated rates						
µg N g^{-1} DW d^{-1}	41.86 (± 5.62)	32.75 (± 4.44)	38.28 (± 1.77)	32.51 (± 9.25)	40.28 (± 8.17)	2.55 (± 0.63)
g N m^{-2} yr^{-1}	27.56 (± 3.8)	32.73 (± 4.11)	30.31 (± 2.33)	15.18 (± 4.33)	4.84 (± 0.97)	2.78 (± 0.67)
kg N ha^{-1} yr^{-1}	275.58 (± 38)	327.33 (± 41.08)	303.11 (± 23.3)	151.8 (± 43.32)	48.41 (± 9.8)	27.84 (± 6.72)

For *G. magellanica*, average estimates using all data from both sample sets are provided as *overall*. Data are means ± SE ($n = 5$). Estimated rates were calculated assuming 100% cover of selected species and assuming continuous nitrogenase activity (poikilohydric organisms will be inactive during dry periods).

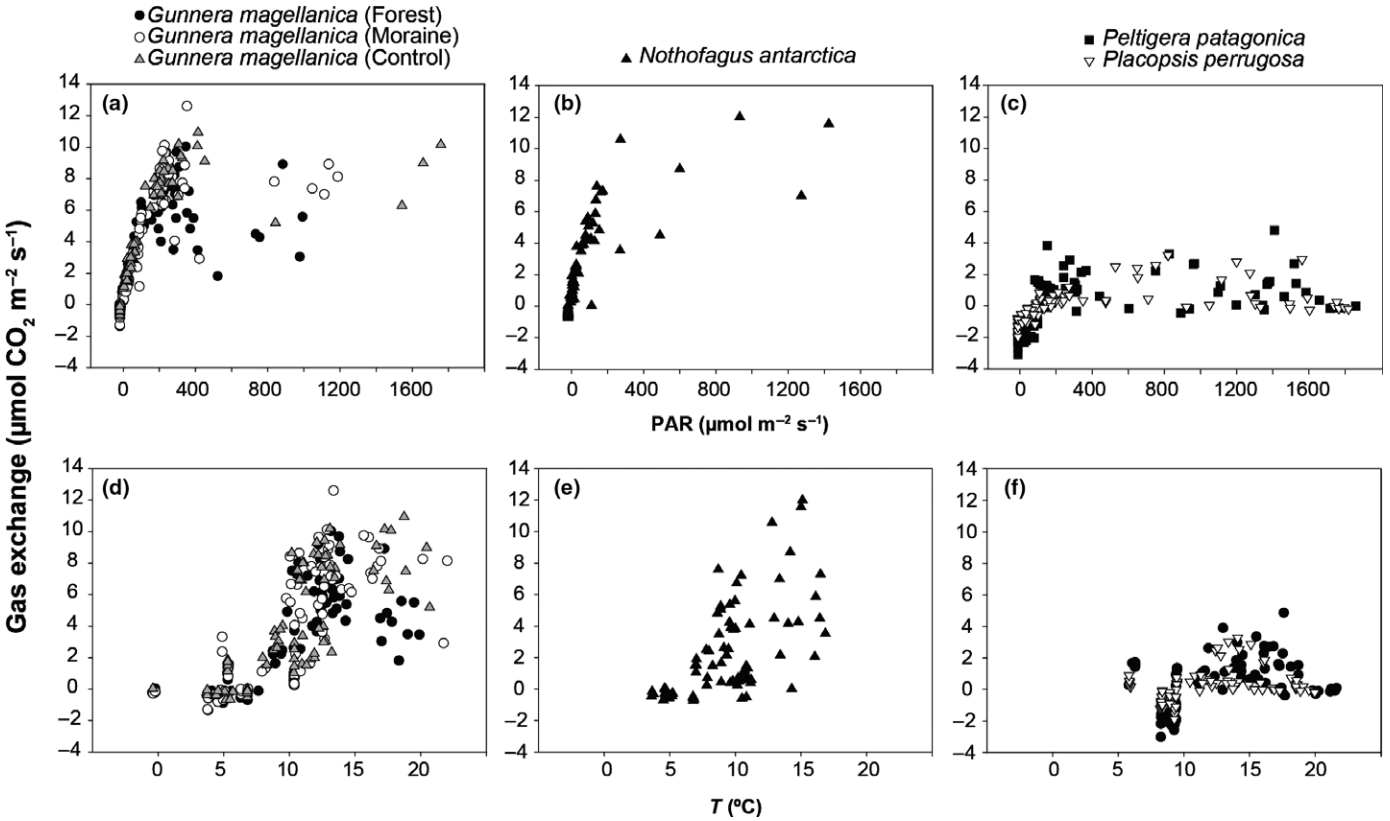

Fig. 3 Gas exchange (respiration, negative values; net photosynthesis, positive) under field natural conditions of light (a–c) and temperature (d–f) for different species considered: *Gunnera magellanica* (a, d), *Nothofagus antarctica* (b, e) and N-fixing lichens (c and f: *Peltigera patagonica*, *Placopsis perrugosa*). $n = 5$ for all cases except for *Nothofagus* tree, where five leaves of the same individual were used. PAR, photosynthetically active radiation; T, temperature.

the Pia Glacier foreland by combining the extraordinarily high N-fixation of this species with the potentially efficient pathways for nutrient acquisition via mycorrhizas in non-N-fixing species.

Nutrient inputs

Recently exposed substrates at the Pia Glacier foreland have very low levels of essential nutrients (Table S3; Arróniz-Crespo *et al.*, 2014). This persists for *c.* 7–10 yr until nutrient stocks (i.e. soil total C, PO_4^{3-} and NH_4^+) start to increase in the soil (Table S3). However, the plants show few signs of nutrient deficiency, as indicated by their high growth rates and also by foliar N : P ratio, which remains almost constant between 10.11 and 12.90 for all species. This is impressive given that the absolute N and P levels in plant tissues differed markedly between species by a factor of up to three. However, for each individual species, levels of both N and P remained almost constant along the chronosequence (Fig. 4a,b) and at relative levels that are expected for the different plant biotypes (e.g. herbaceous, deciduous or evergreen; Larcher, 1995). The N : P ratios are in the range expected for trees (Flückiger & Braun, 2003) but lower than those for herbs (Güsewell, 2004). The N : P ratios are lower than those reported from another glacier foreland nearby (Pérez *et al.*, 2014) but this may be a result of the much slower succession at the latter, 10 times longer to forest, and also a higher P content in all species at Pia Glacier. Plant leaves have their highest N and P contents on the younger surfaces, up to 7 yr (Fig. 4), and leaf $\delta^{15}N$ is also very negative at these initial stages. This suggests that N is initially sourced in meltwater from allochthonous detritus deposited on the glacier surface (e.g. debris coming from surrounding forests above the glacier front, which would be expected to have high leaf N and low $\delta^{15}N$, Fig. S3). This is supported by high potential organic polymer degradation and nitrification by bacterial communities at recently exposed soils (1–7 yr; Fernández-Martínez *et al.*, 2016). There then appears to be a shift from detrital N supply to products of N-fixation, as indicated by the ^{15}N discrimination values which become less negative and reach their maximum at *c.* 10 yr (Fig. 4c). This shift is, almost certainly, initially driven by N-fixing cyanobacteria epiphytic on mosses (i.e. the moss *Ditrichum cylindricarpum*, which can attain instantaneous rates of 283.4 nmol C_2H_4 g^{-1} DW h^{-1}; Arróniz-Crespo *et al.*, 2014) and N-fixing lichens (i.e. *P. patagonica* and *P. perrugosa* with similar high daily N-fixation rates on an area basis (Table 2) or *Placopsis contortuplicata* and *P. stenophylla*; Raggio *et al.*, 2012). Following its early appearance after 4 yr, the main provider of N later in the chronosequence is the higher plant *G. magellanica*, with its unusual cyanobacterial symbiosis. Instantaneous overall mean acetylene reduction rates are 74 nmol cm^{-2} h^{-1} (or *c.* 300 kg ha^{-1} yr^{-1}, Table 2). Although these values should be taken with caution (see Methods S2), they

would exceed those reported for other *Gunnera* species, such as *G. arenaria* (72 kg N ha^{-1} yr^{-1}; Silvester & Smith, 1969), or *G. macrophylla* (12–21 kg N ha^{-1} yr^{-1} at 8–10°C, daylight hours only; Becking, 1976). The N fixation rate found for *G. magellanica* at Pia Glacier is among the highest reported from nodulated phanerogamic species (*Alnus*, *Casuarina*, *Acacia* and *Glycine*; Kellar & Goldman, 1979; Silvester, 1983; George *et al.*, 1988; Rennie *et al.*, 1988; Binkley *et al.*, 1994; Vaishampayan *et al.*, 2001). Nevertheless, strong inhibition in N-fixation is expected during less favorable, colder seasons (Pérez *et al.*, 2017). Our results contrast with previous attempts to provide tentative N-fixation rates of *G. magellanica* in Tierra del Fuego (Troncoso *et al.*, 2013; Pérez *et al.*, 2014, 2017), and this is possibly explained by a negative effect of plant manipulation (Söderbäck *et al.*, 1990) or different methodological approaches (e.g. long incubation periods; Dart & Day, 1971; David & Fay, 1977; Wani *et al.*, 1983). Finally, ARA rates and leaf $\delta^{15}N$ values near zero support *G. magellanica* as being largely reliant on fixed N, suggesting an almost obligate dependency on the symbiosis for its N requirements, as has been proposed for other *Gunnera* species (Osborne *et al.*, 1992).

We can rate the relevance as N contributors in the Pia Glacier foreland in this order: cyanobacteria epiphytic on mosses < lichens < *G. magellanica*. This order is not as much dependent on individual N-fixation rates but on the extent of the organisms in the community (Fig. S1). The loss of open soil surfaces as the herbaceous layer develops and the low amount of rock exposure result in reduced coverage of lichens and mosses especially on older surfaces whilst, by contrast, coverage of *G. magellanica* is extensive at all sites older than *c.* 4 yr. However, both the fate of fixed N and its transfer from N-fixing organisms to other plants depend on different pathways that remain largely unexplored (Lipson & Näsholm, 2001; Persson & Näsholm, 2001).

Mycorrhizas and their transfer role

To achieve the rapid forest succession in Pia Glacier there must be a highly efficient transfer mechanism that moves the fixed N to the non-N-fixing plants as the latter all have high N-contents and growth rates (Fig. 4a). The transfer process appears to be via mycorrhizas because not only do they produce negative $\delta^{15}N$ in host leaf tissues, as found here, but also all the dominant plants on the Pia Glacier foreland are reported to be mycorrhizal, including *G. magellanica* (Table 1). In addition, fungal groups involved in mycorrhiza formation become more abundant when *Nothofagus* spp. and *G. mucronata* establish in the succession (Fernández-Martínez *et al.*, 2017). Many studies have reported that ectomycorrhizal and ericoid mycorrhizal plants in Arctic, alpine or boreal regions are significantly depleted in ^{15}N relative to co-occurring arbuscular mycorrhizal plants (Schulze *et al.*, 1994; Michelsen *et al.*, 1996; Hobbie *et al.*, 2005). The depletion is driven by transfer process of N compounds from the fungus to its host, which leaves the fungus enriched in ^{15}N and the supplied plant depleted (Hobbie *et al.*, 2005; Hobbie & Högberg, 2012). The magnitude of these changes is influenced by the type of mycorrhizal association formed by plants (Hobbie & Högberg, 2012). The $\delta^{15}N$ values of plants that rely exclusively on N-fixation are usually *c.* 0‰ (as occurs for *G. magellanica*), reflecting atmospheric isotopic N values (Handley & Scrimgeour, 1997; Hobbie *et al.*, 2005). A major hindrance to interpreting foliar $\delta^{15}N$ is that multiple N sources can distort isotopic signatures; however, the Pia Glacier foreland is unusual in that, following the removal of any supply from the glacier, N supply is potentially entirely from N-fixation. Furthermore, soil $\delta^{15}N$ in the succession is expected to be low at young successional stages (i.e. *c.* −1‰; Pérez *et al.*, 2014). In addition, low soil N availability is known to limit significant fractionation during direct N acquisition by plant root systems (Craine *et al.*, 2015). Thus, the influence of N transformations (e.g. nitrification) on soil $\delta^{15}N$ is presumably low compared to the effect of N-fixation and N-transfer via mycorrhizas. The application of the formula linking source $\delta^{15}N$ and plant $\delta^{15}N$ to the chronosequence assumes that the proportion of the N coming from *G. magellanica* declines as recycling increases later in the chronosequence (Methods S1) and produces a relatively constant transfer ratio (Tr) at *c.* 0.75 (Fig. 4d). This would indicate that *c.* 75% of N taken up by the mycorrhizas is transferred to the mycorrhizal host. This is a high proportion, probably reflecting the large quantities of N stored in N fixers detritus but it is also highly energetically efficient in terms of carbon supplied by the host (Hobbie & Hobbie, 2008; Hobbie & Högberg, 2012). It is also interesting that the transfer ratio is almost identical for all the plants although the quantities moved to *G. magellanica* must be small. The level of fit with the theoretical equation suggests that mycorrhizal transfer might be the dominant process at Pia and the undetectable nitrification potential of the soils also suggests it might even be the only process (Table S3). The assumptions made in the above calculations include changes in the proportion of recycled and newly fixed N in leaf litter, and the proportion of plant N supplied by the fungus (Methods S1). We feel the values used are reasonable and, considering the end result (i.e. a constant transfer ratio), we suggest that these topics would provide an interesting basis for future research. Although, to date, it has usually been assumed that the fungi utilize N compounds released by soil bacteria (Wu, 2011), the results presented here suggest that the contribution by direct involvement of mycorrhizas might be higher than previously thought.

Involvement of mycorrhizas in the N supply to plants is a more recent development in our understanding of their function (Näsholm *et al.*, 2008, 2013). It has been well known for many years that they are also efficient P suppliers and that plants are dependent on their mycorrhizal symbionts for this function (Jansa *et al.*, 2011; Johnson *et al.*, 2016). It is therefore no surprise that all the plants at Pia Glacier have normal foliar P contents. However, the rapid succession of the vegetation suggests that the plants are adequately supplied with all their nutrients and this includes, as suggested by Zhang *et al.* (2015), supplying magnesium which is absent in the original soils but constant at adequate levels in all the plants (data not shown). This opens the possibility of a much greater role for mycorrhizas in nutrient uptake than has so far been considered.

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Table 3 Averaged values of foliar nitrogen (N) (%), v (%) and $\delta^{15}N$ (‰) obtained from the evaluated plant species for all sampled sites of the Pia Glacier foreland (Tierra del Fuego, Chile).

Species	N	P	N : P	$\delta^{15}N$
Gunnera magellanica (*n* = 28)	3.253 ± 0.053	0.293 ± 0.024	12.765 ± 0.825	−0.314 ± 0.055
Gaultheria mucronata (*n* = 18)	1.054 ± 0.035	0.090 ± 0.012	12.68 ± 1.024	−4.683 ± 0.454
Nothofagus betuloides (*n* = 20)	1.390 ± 0.051	0.190 ± 0.035	10.107 ± 1.107	−4.033 ± 0.410
Nothofagus antarctica (*n* = 16)	2.419 ± 0.119	0.191 ± 0.005	12.901 ± 0.687	−3.958 ± 0.434

Data are means ± SE.

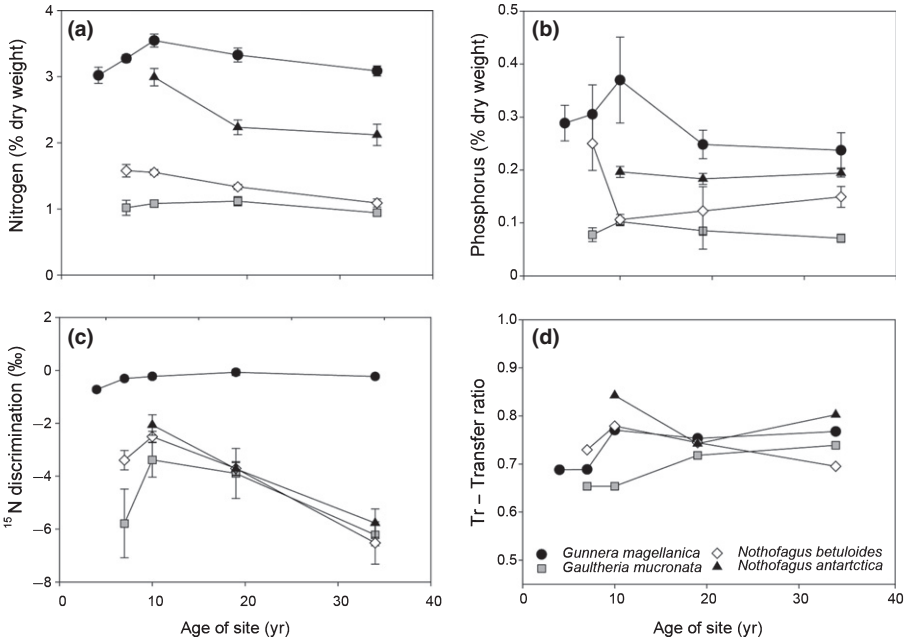

Fig. 4 Leaf elemental and isotopic analysis in selected plant species (see Table 1) along the chronosequence in front of the Pia Glacier, Tierra del Fuego (Chile). (a) Leaf nitrogen (N) content (% DW); (b) leaf v content (ppm); (c) $\delta^{15}N$ patterns; (d) estimated values for Tr (transfer ratio for N from fungus to host) calculated for each species along the chronosequence. Estimations followed these assumptions (see Supporting Information data for a more detailed explanation of assumptions): F is set to 0.7 for all plants except *Gunnera magellanica* which receives its N from the air and has been set at 0.1. Non-N-fixing plants receive N from their mycorrhizas calculated as 90% sourced from *G. magellanica* at 7 yr, 70% at 10 yr, 50% at 19 yr and 30% at 34 yr (mean $\delta^{15}N$ = −0.74, −0.96, −1.92 and −4.39‰, respectively). $\delta^{15}N$ available to *G. magellanica* has been adjusted in the early years to reflect that it is coming mainly from meltwater from the glacier. The values used are estimates only. Values are mean (*n* = 5) ± SE.

Plant photosynthetic status

Both photosynthetic activity and respiration of the herbaceous species *G. magellanica* were similar in range to, but significantly higher than, that observed for the tree species *N. antarctica*. We initially hypothesized that higher N supply would drive higher photosynthetic capacity in *G. magellanica* (Evans, 1989; Reich *et al.*, 1997; Niinemets, 1999; Farquhar *et al.*, 2002; Wright *et al.*, 2004; Shipley *et al.*, 2005). However, the photosynthetic advantage derived from higher N content was much weaker than initially expected, with rates similar to those previously reported from other *Nothofagus* spp. or other species in polar or temperate regions (Larcher, 1995; Martínez Pastur *et al.*, 2007; Muraoka *et al.*, 2008; Albert *et al.*, 2011). The lack of evident differences in photosynthetic activities between N-fixers and non-N-fixers might be a consequence of the absence of N-deficiency in the system, which would minimize the physiological advantage of N-fixers in this habitat, or different N allocation strategies (e.g. less allocated N to Rubisco in *G. magellanica* leaves).

The lower photosynthetic rates of the studied lichens in comparison to the vascular species was not surprising as this has been commonly reported for cryptogams (Larcher, 1995). Previous laboratory analysis indicate that our field measurements were performed under optimum conditions (De los Ríos *et al.*, 2011; Raggio *et al.*, 2012). However, despite their low photosynthetic capacity, cryptogams, which are the dominant organisms at initial stages of primary colonization at Pia Glacier, may explain the slight increment in soil C content during these initial successional stages (Arróniz-Crespo *et al.*, 2014).

Northern/southern hemisphere comparison

The extraordinarily fast primary succession at the Pia Glacier foreland contrasts strongly with the situation reported from other chronosequences in the northern hemisphere. Whilst a mature *Nothofagus* forest has been achieved at Pia Glacier in 34 yr, in Alaska in sites that are also at sea level, a spruce forest became established after *c.* 80–120 yr (Crocker & Major, 1955; Chapin *et al.*, 1994; Alexander & Burt, 1996). In general, vegetation succession to tree cover in northern hemisphere sites takes hundreds of years (Chapin *et al.*, 1994; Sigler & Zeyer, 2004; Jones & Del Moral, 2005; Schmidt *et al.*, 2008; Hodkinson *et al.*, 2009), and thus it is interesting to consider what is different between the two hemispheres. The most obvious difference is that there is no equivalent to *G. magellanica* in the northern hemisphere. N-fixing plants occur at both northern and southern hemisphere

sites, but there is a major contrast in the type and N-fixing ability of these plants. At northern hemisphere sites (i.e. Glacier Bay or Mendenhall Glacier, Alaska), the major N-fixers are *Alnus* species with *Frankia* symbionts which occur later in the succession (Kohls *et al.*, 2003). Some herbaceous or dwarf shrub N-fixers, such as *Dryas drummondii*, appear to impact the succession but their fixation rates seem to be low (Lawrence *et al.*, 1967; Blundon & Dale, 1990). *G. magellanica* not only establishes early in succession but is dominant in the herbaceous layer, excluding most other species, and versatile so that it is present in open and forest environments.

In addition, there is a marked difference in climate between southern and northern hemisphere glacier sites. In particular, although at similar latitudes (59° and 55°, Glacier Bay and Pia, respectively), the winters are much more severe in the northern hemisphere due to the more extensive land masses. Monthly mean temperatures at Pia are always above 0°C (lowest is 1.3°C at Puerto Williams, a nearby coastal town) whilst at Glacier Bay mean monthly temperatures are below zero for 3 months each year and mean minima below zero for 5 months a year (https://wrcc.dri.edu/). Biological N-fixation is strongly negatively impacted by low temperatures (Cleveland *et al.*, 1999; Reed *et al.*, 2011). The daily pattern found here and at other sites showed a night-time depression that was due to low temperature rather than changes in light (removal of leaves, for example, had no effect on the N-fixation rates; data not shown). This has also been shown for other N-fixing symbiotic plants (Eckart & Raguse, 1980; Schweitzer & Harper, 1980). Cyanobacteria are poor performers at subzero temperatures not just for N-fixation but also for photosynthesis (Lange, 1965; Antoine, 2004). Low temperatures are suggested to be the reason why lichens with cyanobacterial photobonts are absent from continental Antarctica (Green *et al.*, 2011).

It is worth comparing the situation in an equivalent southern hemisphere location, namely New Zealand. Some glaciers in the South Islands also terminate at low elevation and run through forested mountains. There are also 10 species of *Gunnera* found in New Zealand but which are mainly small herbs and often rare (WCSP, 2018). There seems to be no equivalent to *G. magellanica* either in size or in ability to dominate the herbaceous layer. Possibly, the strong P limitation characteristic of New Zealand soils (Richardson *et al.*, 2004) is severe enough to limit the performance of *Gunnera* spp., although this is speculative at the moment.

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Author contributions

AB-G, JR, AP, RR, TGAG and LGS designed the study and experimental methodology. Soil and plant material sampling were conducted by LGS, AP and TGAG. Nitrogen fixation analysis were performed by AB-G. Carbon flux measurements were performed by JR, JMB and JV. The manuscript was written by AB-G, JR and TGAG. All authors contributed to the drafts and gave final approval for publication.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Description of the sampled chronosequence in front of the Pia Glacier (Tierra del Fuego, Chile) showing vegetation colonization sequence.

Fig. S2 Temperature control inside and outside incubation bottles.

Fig. S3 Proposed nitrogen uptake and transfer pathways at the Pia Glacier foreland.

Methods S1 Calculations of nitrogen transfers and sources.

Methods S2 Calculation of different bottle headspaces for ARA measurement.

Table S1 Results of PERMANOVA pairwise *post-hoc* comparisons between nitrogenase activity rates obtained for studied species and localities.

Table S2 Results of PERMANOVA pairwise *post-hoc* comparisons between net photosynthesis and respiration rates obtained for studied species and localities.

Table S3 Soil variables measured from soils collected at different ages of exposure along the chronosequence in front of the Pia Glacier (Tierra del Fuego, Chile).

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