Patterns of biological nitrogen fixation during 60 000 years of forest development on volcanic soils from south-central Chile

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Abstract: Biological nitrogen fixation (BNF) is a key process for ecosystem development on new substrates. On young volcanic substrates, the near absence of nitrogen (N) and the presence of available phosphorus (P) in the soil should stimulate the activity of diazotrophic, N-fixing, bacteria. Our main hypothesis is that ecosystem N gain through BNF is tightly coupled to the development of progressive and maximum phases of ecosystem succession, as element contents build up. We investigated ecosystem development in a well-established 60 000-year-old chronosequence in Llaima volcano, south-central Chile. Tree basal areas and total carbon (C), N and P contents in soils showed declining trends over the millennial chronosequence following maximum values between 326 and 3470 years of succession. Maximum rates of BNF (3–6 kg N ha^{-1} year^{-1}) were recorded in the interval from 776 to 3470 years of substrate age, partly associated with high soil C and P contents, and with the lowest denitrification rates in the chronosequence (0.17–0.42 kg N ha^{-1} year^{-1}). Accordingly, over this time interval, there was a positive balance of gaseous N fluxes (5.7–2.8 kg N ha^{-1} year^{-1}), which produced the lowest ^{15}N signal in the surface soil (δ^{15}N = −4.6 ‰). In turn, the first stage of the chronosequence was characterised by low symbiotic N fixation associated with *Racomitrium* moss carpets (0.07 kg N ha^{-1} year^{-1}) that did not compensate for denitrification losses (2.6 kg N ha^{-1} year^{-1}), yielding a negative balance of N gas exchanges. At 3470 years in the chronosequence, BNF rates declined (1.04–2.3 kg N ha^{-1} year^{-1}) and denitrification increased (1.2–2.5 kg N ha^{-1} year^{-1}), leading to N losses and enriching the δ^{15}N signal in the surface soil (−2.4 ‰). Finally, the oldest sites of the chronosequence presented BNF rates that balanced denitrification rates (1.3 kg N ha^{-1} year^{-1}), thus supporting the hypothesis that a net nitrogen budget of zero for N gaseous fluxes characterises the retrogressive phase in this chronosequence.

Keywords: Chile; δ^{15}N; denitrification; ecosystem development; Llaima volcano; primary succession; southern temperate forests

Introduction

During the course of ecosystem development, over millennial timescales, three distinct successional phases have often been recognised in the absence of rejuvenating disturbances: progressive, maximal and retrogressive (Peltzer et al. 2010). These successional stages describe the increase, the peak, and then the decline of ecosystem productivity associated with the build-up and the subsequent loss of limiting elements, particularly phosphorus (P), over long periods of soil development (Walker & Syers 1976; Vitousek & Farrington 1997; Wardle et al. 2004; Peltzer et al. 2010). Ecosystem changes during a volcanic soil chronosequence in Hawai'i were driven by high inorganic P availability in primary minerals during the early stages of succession, followed by a build-up of organic P at intermediate successional stages, and culminating in a sharp decline of both forms of P in older substrates (Crews et al. 1995).

Biological nitrogen fixation (BNF) plays a key role at the onset of ecosystem development after major disturbances, or on denuded surfaces, when soil carbon (C) and nitrogen (N) stocks are greatly depleted or exhausted, and rock-derived elements may be available. BNF is the process by which unreactive atmospheric nitrogen (N$_2$) is taken up by symbiotic or free-living diazotrophic bacteria and transformed into bio-available ammonium by the action of the enzyme nitrogenase. In nature, free-living, N-fixing bacteria thrive on carbon-rich substrates that provide energy for N reduction. Suitable substrates for free-living diazotrophs include forest litter, organic soils, and coarse woody debris (Cleveland et al. 1999). Free-living bacteria can also be phototrophic, living on protected and intricate surfaces of liverworts and mosses (Adams & Duggans 2008). Phototrophic bacteria can also live in symbiosis with non-vascular plants such as mosses, liverworts, hornworts, and lichens. Among the most widespread phototrophic diazotrophs are blue-green algae (cyanobacteria), particularly in the genus *Nostoc* (Rai et al. 2000).

Both C and P availability can limit the activity of free-living N fixers early in succession, because of the high C demands as a source of energy for heterotrophic activity, and the high ATP requirement for cellular N fixation by both autotrophic and heterotrophic bacteria (Reed et al. 2007, 2011). Fertilisation studies have often shown a temporal shift from N to P limitation during the course of succession on volcanic soils (Vitousek & Farrington 1997). Accordingly, N fixation by forest-floor diazotrophs was much lower in old soils than

in younger volcanic soils (Crews et al. 2000). Likewise, in a 15-year study, P fertilisation applied to the floor of Hawaiian forests increased the abundance of epiphytic cyanolichens, hence strongly enhancing BNF (Benner & Vitousek 2012). That study suggests that increased P limitation during the retrogressive phase of succession could also cut down symbiotic N fixation in the epiphytic layer.

Biogeochemical theory predicts that the chemical forms of biologically available nutrients (accessible for plant or microbial uptake) in the soil solution are subjected to massive gaseous and hydrological losses after major disturbances. Such losses tend to decline during the successive phases of ecosystem development if disturbances are low or absent. This happens because limiting elements are increasingly retained within the ecosystem and accumulated in plant and microbial biomass, up to an equilibrium point during late succession, where element outputs (losses) equal inputs or gains (Vitousek & Reiners 1975). The validity of this traditional equilibrium view of succession is restricted to biologically controllable element pools in the soil, which in the case of N are the product of two main input and output processes, biological N fixation and denitrification (Hedin et al. 2003; Menge 2011). In this regard, studies of millennial chronosequences on volcanic soils in Hawai'i have documented hydrologic N losses and denitrification rates (Hedin et al. 2003) by recording an increase in the ^{15}N isotopic signal in ancient successional soils (Martinelli et al. 1999). Such N losses in the absence of significant rejuvenating disturbances characterise the retrogressive phase of ecosystem development. Accordingly, substrate age is a key variable that could affect ^{15}N accumulation or depletion patterns during succession. In the long-term chronosequences that have been studied, older soils tend to exhibit higher δ^{15}N values than younger or early successional soils that have lower N losses (Vitousek et al. 1989; Brenner et al. 2001; Hobbie & Högberg 2012).

A study of a millennial soil chronosequence on Llaima volcano, Chile (Gallardo et al. 2012), showed that the total contents of C, N and P were extremely low in young volcanic soils on historical lava flows (less than 60 years old). For these elements, a progressive accumulation phase followed, reaching a peak between 320 and 770 years of ecosystem development, which was followed by strong declines in C, N, and P in soils older than 40 000 years BP, which could be interpreted as the retrogressive phase of ecosystem development (Gallardo et al. 2012).

Accepting that C and P are both limiting elements for the occurrence of BNF in soils, and particularly P for epiphytes, the present study addresses the hypothesis that the occurrence of N fixation during ecosystem succession should be coupled to the changing patterns of soil contents of these two elements through the chronosequence. Patterns of BNF and element content in soils should change in concert through the hypothetical progressive, maximal, and retrogressive phases of ecosystem development on volcanic soils. Moreover, these coupled trends should be more evident for heterotrophic free-living soil bacteria, as they depend more directly on soil nutrient status than autotrophic cyanolichens in the epiphytic layer. Based on current knowledge of successional patterns on volcanic substrates (Crews et al. 1995), the progressive and maximal phases of ecosystem development should be associated with a positive N balance due to increased retention within the ecosystem, and because N inputs via BNF should be higher than hydrologic and gaseous N losses, which are larger in early- and mid-succession. In turn, the hypothetical retrogressive phase should be characterised by a negative ecosystem N balance, as losses via denitrification and hydrologic pathways tend to increase in older ecosystems. Due to the higher N losses expected for the retrogressive phase, we expect a concomitant increase in the ^{15}N signal of soils. To test these hypotheses, we estimated the basal area of woody plants, analysed the soil concentrations of C, N and P and δ^{15}N ratios, and determined rates of N fixation and denitrification at volcanic sites with different soil age along a chronosequence near Llaima Volcano at the western slopes of the Andes in south-central Chile.

Study site and methods

One of the most active volcanoes in southern South America is the Llaima volcano (38º41′ S; 71º43′ W) in south-central Chile, for which more than 50 significant eruptions have been historically documented since 1640. Geological records of volcanic activity in this area date back to the late Pliocene (Naranjo & Moreno 2005) and have been fairly continuous. The lavas from Llaima volcano consist of basaltic andesite without significant differences in the chemical composition of rock minerals among eruptions. The present study was conducted on the eastern flanks of Llaima volcano in Conguillío National Park, where several volcanic outflows of different ages are found in the area at a spatial scale of c. 90 km^2 (Fig. 1). We selected seven different volcanic soil formations, which have developed vegetation for different periods of time from the mid-Pleistocene to the present (Fig. 1; Table 1). These areas provide a representative sample of stages of ecosystem development on volcanic substrates. The dominant vegetation is broad-leaved rainforest, under a temperate climate, with Mediterranean influence due to the drier summer months (December–February) and because c. 70% of the 1945 mm of mean annual precipitation falls in the autumn and winter months. Meteorological records maintained for 43 years, 22.5 km north of the study site, indicate an annual average temperature of 8.6°C (di Castri & Hajek 1976).

In the Andes of south-central Chile, the canopy of old-growth rainforests is dominated by different evergreen and deciduous species of *Nothofagus* trees, which constitute mixed communities with the broad-leaved conifer *Araucaria araucana*. The native bamboo, *Chusquea culeou,* dominates the forest understorey. Mid-elevation *Nothofagus* forests are species-rich and productive, with a multi-aged structure and complex vertical profile. Forests often regenerate after catastrophic disturbances such as earthquake-triggered landslides on steep slopes, and volcanism (Pollmann 2003). A diverse flora of epiphytes is found in the forests, with the presence of foliose cyanolichens such as *Pseudocyphellaria*, *Nephroma* and *Peltigera,* all of which have symbiotic associations with N-fixing *Nostoc.* Two shrub species, *Discaria chacaye* and *Colletia spinosissima*, both Rhamnaceae, which are present in the early and late stages of succession, respectively, have actinorhizal associations with N-fixing *Frankia*. Further description of vegetation development patterns on volcanic substrates is provided by Gallardo et al. (2012).

Assessment of basal areas and determination of soil C, N, and P

To isolate the variable successional time from other confounding effects on soil pedogenesis, such as exposure to westerly winds and topography, we restricted the study to seven sites on the eastern slopes and mid-elevation areas of

Figure 1. Map of the study area. Abbreviations (after Naranjo & Moreno 2005) indicate volcanic soil ages, which are also given in Table 1. (Modified after Gallardo et al. 2012.)

Table 1. Site codes and substrate ages (after Naranjo & Moreno 2005) for the different ecosystem developmental stages (numbered 1 to 7, column 2) in the Llaima volcano chronosequence. Site elevations above sea level, soil carbon:nitrogen (C:N) ratios, and available contents of ammonium (NH_4), nitrate (NO_3) and phosphorus (P) in surface soils of each study site are shown in columns 4–8. Values are means ($\pm$ 1 SE) (n = 6 per site).

Site code	Developmental stage	Soil age (years BP)	Elevation (m a.s.l.)	Soil C:N	NH_4-N (mg/kg)	NO_3-N (mg/kg)	Available P (mg/kg)
LlH-1	1	260	875	15.7 (0.91)	6.7 (1.7)	3.4 (0.6)	5.8 (0.3)
Llf3	2	326	1002	21.05 (0.5)	150 (34)	22.7 (6.0)	32.8 (3.1)
Llf2	3	776	1148	27.11 (0.77)	10.5 (4.3)	5.9 (0.5)	24.1 (5.2)
Llf1	4	3470	1196	25.87 (1.03)	18.5 (4.7)	10.0 (3.0)	12.5 (1.6)
Llic	5	12 760	1266	29.93 (1.96)	23.5 (5.5)	6.7 (0.8)	31.7 (5.0)
Ll2	6	40 000	1016	25.83 (0.93)	31.7 (5.2)	7.7 (1.6)	19.1 (2.0)
Ll1	7	60 000	940	22.39 (0.67)	55.2 (4.8)	9.0 (2.5)	13.3 (0.8)

the Llaima volcano (Fig. 1). Unfortunately, selection criteria reduced the possibility of finding a larger number of replicate sites with the same geological formation and substrate age. In the face of the inevitable problem of replication due to sampling of one site per substrate age, we were more interested in documenting long-term patterns of ecosystem change through the chronosequence than in assessing specific differences among sites.

During the spring of 2009, in each of the seven study sites selected on the Llaima volcano, we established three circular plots of 13-m diameter (0.053 ha), separated from each other by at least 100 m in order to minimise, as far as possible, the homogenising effect of pseudo-replication. Within each circular plot, all trees > 5 cm in trunk diameter at breast height (1.3 m from the ground) were measured to estimate tree basal areas per species and site, which were used as a proxy for above-ground biomass. During spring of 2009 and 2010, two soil

samples from the surface soil (A_h horizon, 0–10 cm depth) were taken from within each of the three plots, at random locations separated by at least 10 m from each other, making a total of six soil samples per site. Soil samples were taken with a shovel and put into ziplock bags for transportation to the laboratory.

A subsample of each soil sample was sieved (2-mm mesh size), ground and dried for determination of total C and N by flash combustion in a Carlo Erba NA 2500 Elemental Analyser (Thermo Fisher Scientific, Milan, Italy). Total phosphorus from soil samples was extracted in a concentrated sulphuric acid – water – peroxide solution in a Digesdahl® Digestion apparatus (Hach, Colorado, USA) and determined colorimetrically using molybdenum-blue (Steubing & Fangmeier 1992). Plant-available P was extracted from sieved fresh soils by the Calcium-Acetate-Lactate method (CAL) and determined colorimetrically by the molybdenum-blue method (Steubing &

Fangmeier 1992). Additional subsamples of sieved fresh soils were extracted in a 0.021 mol/L $KAl(SO_4)_2$ solution (1:4) to determine ammonium and nitrate concentrations by means of fractionated steam distillation (Pérez et al. 1998). This method has been used as a standard for acidic forest soils (Runge 1974; Gerlach 1978), because colorimetric determination methods can interfere with the high concentrations of coloured organic acids in these soil types.

Samples for N fixation

Nitrogen fixation was estimated using the acetylene reduction assay (ARA, see below) after laboratory incubation of mixed samples of recently fallen litter collected from the same two sampling points per plot that were used for soil sampling at the seven study sites during spring 2009, 2010 and 2011. Epiphytic foliose lichens were sampled from the main trunks and branches of randomly selected host trees in forests from successional stages 2–7 (Table 1), during spring 2010 and 2011, and then incubated in the laboratory to estimate N fixation. Soil carpets of the pioneer moss *Racomitrium lanuginosum* were sampled from surfaces of historical lava flows belonging to developmental stage 1 (Table 1). Additionally, in each of the two sampling points per circular plot, two cores from surface mineral soil (A_h horizon, 0–5 cm soil depth) were taken using 80-cm^3 PVC tubes and put into ziplock bags ($n = 6$ per study site) for laboratory incubation and ARA. Additionally, during the spring of 2012, in situ ARAs were conducted at seven randomly selected points in each study site, for comparison with lab estimates. For field measurements, samples from the O_l and A_h soil horizons, and epiphytes, were collected and incubated separately.

To estimate the biomass of foliose lichens growing as epiphytes in each forested study site, we selected two trees of each diameter class: 5–15 cm, 15–25 cm, and >25 cm ($n = 6$ per site). A 15 cm wide low-density polyethylene belt was placed around the tree trunk at 1.3 m above the ground and at the base of each tree. All foliose lichens growing within these belts were removed and put into zip lock bags. Samples of foliose lichens growing on trees were taken to the laboratory and a subsample was dried at 70°C for at least 48 h for dry weight determination. Epiphyte dry weights were first calculated on a per area basis (i.e. within each 15-cm-wide belt), then averaged over both tree heights to obtain a final biomass value per tree and six samples of epiphytic biomass per site.

In each of the two soil sampling points per circular plot, the plant necromass of the O_l soil horizon was determined by collecting fine litter from 15 × 15 cm squares ($n = 6$ per successional site). The same method was used to collect samples of *Racomitrium lanuginosum* from historical lava flows. Samples were then taken to the laboratory and dried at 70°C for at least 2 days for dry weight determination. Possible actinorhizal associations of vascular plants such as *Discaria* and *Colletia* were not investigated in the present study.

Biological nitrogen fixation and denitrification measurements

To focus on ecosystem development and to control for seasonality and small-scale spatial variation in substrate conditions, we conducted all assays in the laboratory at a constant temperature of c. 22°C (a typical summer temperature in the study area). Both epiphytic lichens and litter samples were hydrated prior to the assays to c. 60–70% water content. During the sampling periods (austral spring), several consecutive rainless days may occur because of the Mediterranean climate

influence, and hence water contents of the litter layer and epiphytic lichens can often drop to less than 25% in the study sites (Appendix 1). Under such conditions nitrogenase activity can be drastically diminished (Gundale et al. 2009; Jackson et al. 2011), especially in contrast to laboratory incubations. Therefore, an additional experiment was performed in situ in spring 2012 to assess how estimates of nitrogenase activity based on laboratory incubations differed from the actual values in the field. Field assays were conducted during November 2012 (austral spring). Seven random samples of three communities of N-fixing organisms (epiphytic foliose lichens, plant litter, and mineral soil) were taken from each of the seven stands in the chronosequence. Substrate samples were deposited inside hermetic 500-ml glass jars for acetylene reduction assays (see below) and placed on the forest floor. One gas sample per jar was taken every day for two consecutive days and injected inside 4-ml BD Vacutainers® for subsequent lab analysis.

Estimates of BNF using ARA followed the procedures described by Myrold et al. (1999). In the laboratory, one homogenised sample per site, representing each of two N-fixing community types (litter and epiphytic lichens), and six soil cores per site were placed for up to 2 days inside hermetically-sealed 500-ml glass jars containing a mixture of air and acetylene (10% v/v). An additional subsample per substrate was incubated without acetylene to serve as control. Gas samples were analysed for ethylene production using a Shimadzu gas chromatograph GC-8AIF (Tokyo, Japan) equipped with a steel column filled with Porapak™ N adsorbent (Supelco®, 1 m × ¼″ stainless steel, 80–100 mesh) (Sigma-Aldrich) and an FID detector. Ethylene concentration in gas samples was determined from a calibration curve by diluting a 100-ppm ethylene standard from SCOTTY® analysed gases (Sigma-Aldrich). Acetylene reduction activity was estimated from the slope of the linear fit of the ethylene production curve over 2 days of incubation in 500-ml headspace and related to sample dry weight. Annual rates of N fixation per substrate type were estimated assuming a conversion factor of one-third of the acetylene reduction rate, multiplied by the sample biomass (Zuberer 1998).

Denitrification rates were estimated using the acetylene inhibition assay in intact soil cores (Groffman et al. 1999). Soil samples ($n = 6$ per successional site) were placed in 500-ml hermetic glass jars and incubated for 6 h under a 10% v/v acetylene atmosphere at c. 22°C, with the average soil moisture in the field (c. 60% water content for most sites, except for the youngest substrate in the chronosequence, with c. 30% soil moisture). Gas samples were taken after 2 h and 6 h. The N_2O concentration in gas samples was determined using a Shimadzu gas chromatograph (Tokyo, Japan) equipped with a Porapak Q™ (80–100 mesh) column (Sigma-Aldrich) and electron capture detector. The calibration curve was prepared by diluting 1-ppm nitrous oxide standard from SCOTTY® analysed gases (Sigma-Aldrich). Denitrification rates were estimated from N_2O-N concentration differences between samples taken after 2 h and 6 h of incubation and related to an area basis. All laboratory analyses and assays were performed at the Biogeochemistry Laboratory, Pontificia Universidad Católica de Chile.

A partial ecosystem N budget, restricted to only gaseous exchanges of N driven by soil microbial processes, was estimated for each of the seven successional sites. Calculations considered inputs to the ecosystem via BNF on the basis of N fixation rates measured for the different N-fixing community types in lab assays, minus gaseous N outputs via denitrification from soil samples.

Natural abundances of ^{15}N in soils of the seven successional sites were determined from one soil sample per circular plot ($n = 3$ per site) and expressed as $\delta^{15}N$ ($^o/_{oo}$), according to the following equation:

$$\delta^{15}N = 1000([R_{sample}-R_{air}]/R_{air}), \text{ where } R = {}^{15}N/{}^{14}N.$$

Soil samples (from the first 10 cm) for determination of isotope ratios were taken in November (austral spring) of 2010, sieved (2-mm mesh size), dried at 70°C, and then ground. Determinations were made in a Thermo Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) at the Department of Geobotany, University of Trier, Germany. The average standard deviation of $\delta^{15}N$ (versus air N_2 as standard) is $<0.05^o/_{oo}$.

Statistical analyses

For 2009 and 2010, data are averages of two samples (either for litter, soil or epiphytes) taken from each of three circular plots per successional site, making a total of three mean values per site in the chronosequence. One-way ANOVAs were used to assess the effect of ecosystem developmental stage (1–7), on the following dependent variables: total tree basal area, total contents of soil C, N and P, total BNF (adding all N-fixing community types tested), denitrification rates from soils, partial budget for gaseous N fluxes, and $\delta^{15}N$ (isotopic ratios) in soils. Due to the low number of replicates per site in the chronosequence ($n = 3$), ANOVAs with 1000 permutations were applied (Legendre & Legendre 1998). A posteriori Tukey's tests were used to assess the significance of the differences among successional sites. A confounding effect of elevation on the chemical properties of soils was discarded as the coefficient of determination between elevation and soil age was 0.025.

To assess the effect of three main N-fixing community types (litter layer: O_l horizon; mineral soil: A_h horizon; foliose lichen epiphytes) on the rates of BNF in each successional stage, one-way analysis of variance (ANOVA) was applied after Box–Cox transformation of the data, to correct for variance heterogeneity when needed (Cheng & Lockhart 1997). A posteriori Tukey's tests were used to assess the significance of the differences among N-fixing community types. One way-ANOVA was used in order to test the null hypothesis that lab and field means of acetylene reduction activity in each of the three N-fixing community types were equal. In the case of the epiphytic community when variances were heterogeneous and with an unequal sample size, we applied the Welch test (Quinn & Keough 2002). Probabilities of 0.05 or less were considered statistically significant. All statistical analyses were conducted using R 2.5.1 software (R Development Core Team 2012). Permutations were performed with function "anova.1way", freely available (not included in a package) on http://adn.biol. umontreal.ca/~numericalecology/Rcode/.

Results

Stage 1 of ecosystem development is dominated by isolated individuals of the conifer *Austrocedrus chilensis*, the Proteaceae *Lomatia hirsuta* and the evergreen *Nothofagus dombeyi* (Fig. 2). During stage 2 a closed canopy is exclusively dominated by the deciduous species *Nothofagus alpina*, and towards more advanced stages, *Araucaria araucana* codominates either with the deciduous *Nothofagus antarctica* in the understorey at stage 3 or with the evergreen *N. dombeyi* in the upper canopy at stage 4 at the maximum stage of total tree basal area (Fig. 2). The deciduous *Nothofagus pumilio* and the evergreen *N. dombeyi* codominate with *A. araucana* at developmental stage 5 (Fig. 2). Finally, during developmental stages 6–7, several evergreen angiosperms such as the Asteracean tree *Dasyphyllum diacanthoides*, *Lomatia hirsuta*, *Raukaua laetevirens* and *Aristotelia chilensis* dominate the understorey
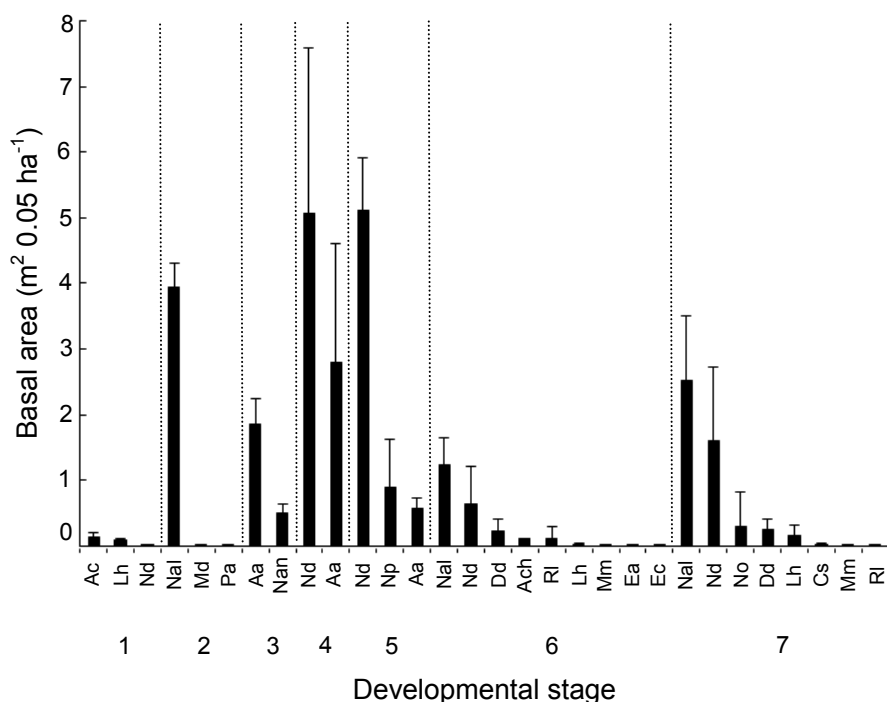
Figure 2. Changes in basal area of woody species present in the seven ecosystem developmental stages in the 60 000-year successional chronosequence of Llaima volcano in Conguillío National Park, Chile. Ecosystem developmental stages (1 to 7) are defined in Table 1. Plotted values are means ($n = 3$ plots per site) ± one standard deviation. Ac: *Austrocedrus chilensis*, Lh: *Lomatia hirsuta*, Nd: *Nothofagus dombeyi*, Nal: *Nothofagus alpina*, Md: *Maytenus distichia*, Pa: *Prumnopitys andina*, Aa: *Araucaria araucana*, Nan: *Nothofagus antarctica*, Np: *Nothofagus pumilio*, Dd: *Dasyphyllum diacanthoides*; Ach: *Aristotelia chilensis*, Rl: *Raukaua laetevirens*, Mm: *Maytenus magellanica*, Ea: *Escallonia alpina*, Ec: *Embothrium coccineum*, No: *Nothofagus obliqua*, Cs: *Colletia spinosissima*.

with *N. alpina*, the deciduous *N. obliqua* and *N. dombeyi* in the upper canopy, resulting in higher tree species richness (Fig. 2).

The initial soil developmental stage, a 260-year-old lava flow in the Llaima volcano chronosequence, presented the lowest soil C:N ratio and the minimum available N and P concentrations in surface soils (Table 1). We found statistically significant differences among successional sites, representing different ecosystem developmental stages in the chronosequence, for all the dependent variables measured: tree basal areas, total soil carbon contents, N and P concentrations in soils, total N fixation, denitrification rates in soils, balanced N inputs from gaseous exchanges, and $\delta^{15}N$ in soils (Table 2).

Maximum tree basal area occurred at stage 4 of forest ecosystem development in this chronosequence (Fig. 3a), which is represented by a substrate age of 3470 years. Total soil C (Fig. 3b), N (Fig. 3c), and P (Fig. 3d) contents reached maximum values at ecosystem stages 2 and 3, corresponding to substrate ages of 326 and 776 years respectively. Soil contents of all three elements declined in older soils except for a slight increase in the latest successional stage, but only for N and P.

Overall, BNF showed an increasing trend from early succession up to an age of 776 years of stand development (ecosystem stage 3). After this maximum, BNF declined in stands older than 3470 years (Fig. 4a) and remained rather low in advanced stages of the chronosequence. In all ecosystem stages in the chronosequence, there were significant differences among N-fixing community types (Table 3), but the most important N-fixing community type was not the same in all ecosystem stages (Fig. 4b). For example, in ecosystem stage 3, when BNF reached the highest rates, lichen epiphytes with symbiotic blue-green algae presented higher rates of N fixation than forest soil and litter (Fig. 4b). In most other stages in the chronosequence, however, characterised by lower rates of BNF, mineral soil samples presented the highest rates of N fixation. All N-fixing community types presented similar trajectories across the chronosequences, with maximum values for ecosystem stage 3 (Fig. 4b) and low values for other stages, except for foliose lichen epiphytes that presented a secondary peak in old stands.
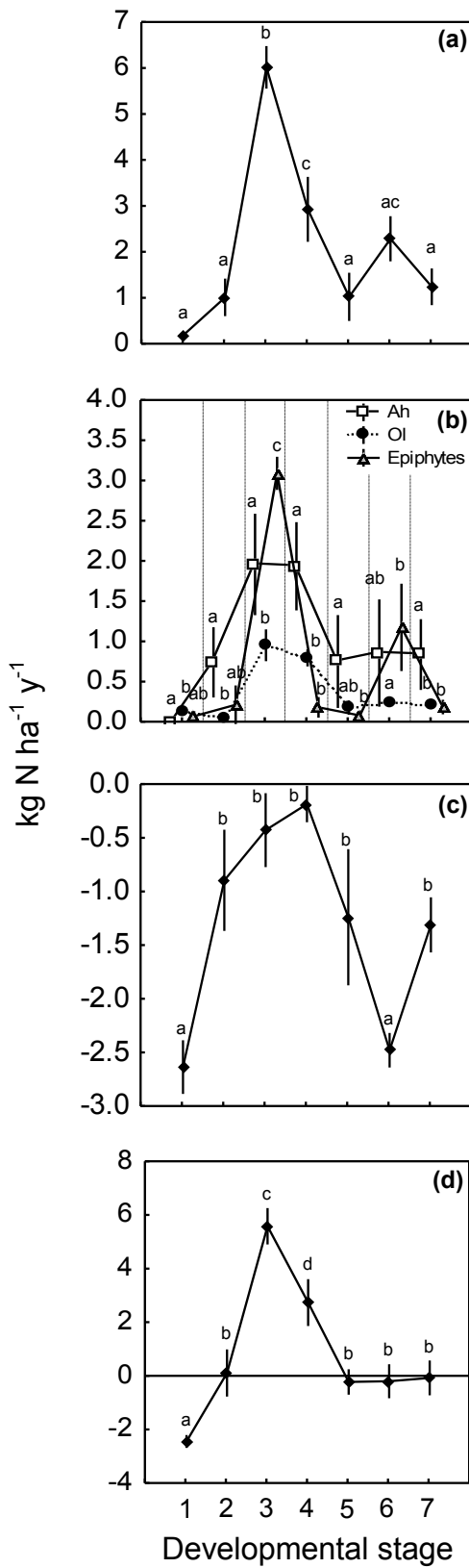
Denitrification rates in forest soils were maximal for the youngest stage in the chronosequence (most negative values representing higher gaseous N losses, Fig. 4c). One of the oldest ecosystem stages, stage 6, had also high rates of denitrification

Table 2. Results of one-way ANOVA to assess the differences in tree basal areas, soil contents of total carbon (C_t), nitrogen (N_t) and phosphorus (P_t), denitrification rates, balance of gaseous fluxes of N, and $\delta^{15}N$ signal in surface soils for seven ecosystem stages from the Llaima volcano chronosequence. Analyses indicated significant differences among stages in the chronosequence for all dependent variables.

Dependent variables	$F_{6,14}$	P
Basal area	132.6	<0.0001
C_t	26.31	<0.0001
N_t	41.2	<0.0001
P_t	29.19	<0.0001
Total BNF	46.17	<0.0001
Denitrification	18.23	<0.0001
N budget	41.2	<0.0001
$\delta^{15}N$	4.01	0.0171
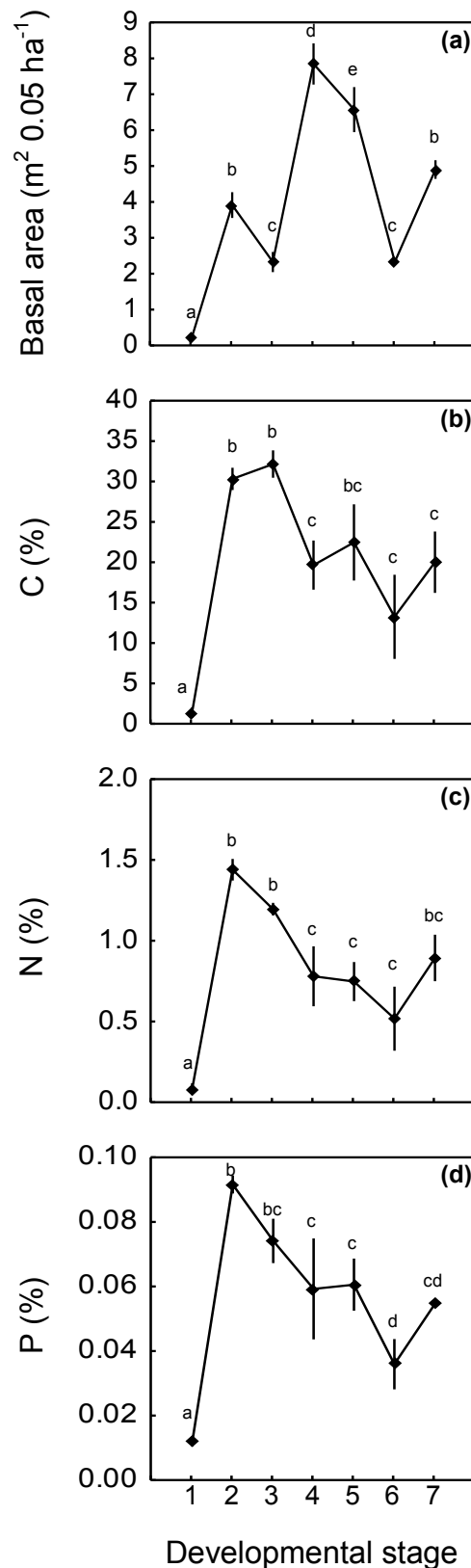
Figure 3. Tree basal area and total element concentrations of seven ecosystem developmental stages in the 60 000-year successional chronosequence of Llaima volcano in Conguillío National Park, Chile: (a) tree basal area, (b) soil carbon, (c) soil nitrogen, (d) soil phosphorus. Different letters above data points indicate significant differences among ecosystem developmental stages (Tukey's test, *P* < 0.05). Plotted values are means (*n* = 3 per site) ± one standard deviation.

(Fig. 4c). The partial budget for N gaseous fluxes of nitrogen, after subtracting denitrification losses from BNF inputs, was distinctly negative for stage 1 in the chronosequences (Fig. 4d), on the youngest volcanic substrate. A positive balance regarding gaseous N fluxes characterised chronosequence stages between 326 and 3470 years, with the most positive value recorded for ecosystem stage 3, when BNF was maximal. For the three oldest stages in the chronosequence, the net gaseous N flux was slightly negative or zero (Fig. 4d).

Field rates of acetylene reduction measured for foliose lichen epiphytes or for litter samples were generally lower than rates measured in the laboratory (Welch $t = -5.84$, d.f. $= 41.005$, $P < 0.0001$; $F_{1,80} = 63.8$; $P < 0.0001$, respectively), with the exception of ARA values for the A_h soil horizon, which were similar in the lab and in the field ($F_{1,81} = 1.956$; $P = 0.166$) (Appendix 2). The litter layer of ecosystem stages 4 and 5 (3470 years and 12 760 years of substrate age, respectively) presented the highest acetylene reduction activities measured in the field. These substrate samples also presented the highest moisture contents (Appendix 1).

The ^{15}N isotopic signal determined for surface soils in the Llaima volcano chronosequence showed a declining (yet statistically insignificant) trend for ecosystem stages 1–3 and remained low at the intermediate stages 3–5 ($\delta^{15}N = -4.7‰$ to $-4.5‰$). In the surface soils of the oldest stage 7 of the chronosequence, ^{15}N was significantly enriched ($\delta^{15}N = -2.4‰$) in relation to stage 3 (Fig. 5).
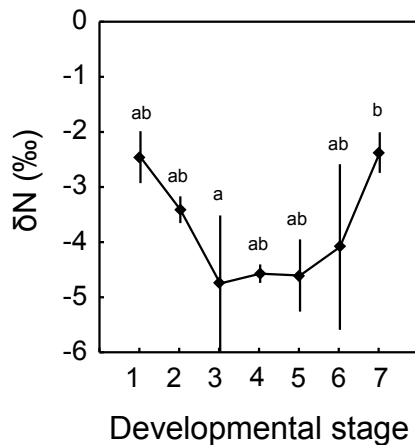
Discussion

Changes in tree basal area and soil element concentrations

A significant decline in tree basal area took place in the two oldest ecosystem stages of the Llaima volcano chronosequence (substrates 40 000–60 000 years old), which suggests a post-volcanism soil development pattern that culminates in a

Table 3. Results of one-way ANOVA to assess the effects of different N-fixing community types (litter, mineral soil, foliose lichen epiphytes) on rates of biological nitrogen fixation for each of seven developmental stages in the Llaima volcano chronosequence.

Chronosequence stage	$F_{2,6}$	P
1	7.38	0.02
2	7.07	0.03
3	21.48	0.002
4	21.67	0.002
5	7.13	0.03
6	5.76	0.04
7	12.52	0.007

Figure 4. (a) Annual rates of BNF (all N-fixing community types) across all the developmental stages (1 to 7) in the chronosequence. (b) Annual rates of BNF for the litter layer (O_l soil horizon), for the mineral horizon (A_h soil horizon), for *Racomitrium lanuginosum* moss carpets (ecosystem stage 1 only), and for epiphytic foliose lichens (ecosystem stages 2–7). (c) Denitrification rates in the soil. (d) Partial ecosystem nitrogen balance (gaseous fluxes only) expressed as the difference between overall rates of BNF (gains) and denitrification (losses) for different ecosystem stages in the long-term chronosequence studied. Different letters above data points in plots (a), (c) and (d) indicate significant differences among developmental stages (Tukey's test, $P < 0.05$). For plot (b), different letters above data points for a given chronosequence stage indicate significant differences in N fixation rates among N-fixing community types. Values are means ($n = 3$ per site) ± one standard deviation.

Figure 5. Natural abundance of ^{15}N in surface soils of seven developmental stages in the long-term chronosequence from the Llaima volcano. Different letters above data points indicate significant differences among ecosystem stages (Tukey's test, $P < 0.05$). Values are means ($n = 3$ samples per site) ± one standard deviation.

retrogressive phase, following the initial progressive phase characterised by the accumulation of biomass or basal area. Such a pattern of succession should be accompanied by late-successional depletion or reduction of nutrient availability in long-term chronosequences (Peltzer et al. 2010). Along our ecosystem development gradient, evergreen and deciduous *Nothofagus* species are indistinctively present; however, during the maximum basal area, at stage 4, deciduous species are absent. During the retrogressive phases 6 and 7, the conifers *Araucaria araucana* and *Austrocedrus chilensis* are completely lacking, although several evergreen angiosperm species are abundant in the understorey, resulting in higher tree species richness during retrogression. Comparing several long-term chronosequences, Wardle et al. (2008) documented a general decrease in tree species richness during retrogression, when P contents in soils are lower. On the other hand, high tree diversity in P-poor soils has been found in highly diverse tropical forests, which is explained by the high P use efficiency of functional groups (Kitayama 2005). In fact evergreen angiosperms that become dominant in the understorey of retrogressive phases at the Llaima chronosequence have leaves with higher N:P and C:P ratios (N:P = 15.9 ± 3.8, C:P = 613 ± 132) than deciduous species (N:P = 10.9 ± 1.09, C:P = 163 ± 50) and conifers (N:P = 10.6 ± 1.1, C:P = 519 ± 82) suggesting a higher P limitation and higher P use efficiency for evergreen angiosperms that appear during retrogression (Gallardo et al. 2012).

In the Llaima volcano chronosequence, we recorded a strong decline in total contents of soil C, N and P starting from ecosystem stage 4 (3500 years old), which may be taken as evidence for rapid retrogression occurring from mid- to late stages of succession on these volcanic soils. Peltzer et al. (2010) document that successional patterns leading to retrogression can occur over quite different timescales, depending on ecosystem type, overall climate, geologic substrate origin, and disturbance regimes. Accordingly, maximum tree basal area that was recorded at ecosystem stage 4 (3500 years old) in the Llaima volcano chronosequence obviously has developed under conditions of moderate to strong nutrient limitation (Fig. 3).

Evidence from this successional study of the Llaima volcano chronosequence in south-central Chile is consistent with data from other studies on tropical and temperate forest successions on volcanic soils (Torn et al. 1997; Turner et al. 2007; Peña-Ramírez et al. 2009). In these examples of retrogressive ecosystem development, the marked declines in soil C and P in volcanic soils have been explained by declining contents of the non-crystalline minerals, i.e. amorphous allophanic soil minerals, which has been shown to play a decisive role in C (Matus et al. 2006) and P occlusion in Chilean Andisols (Borie & Zunino 1983), due to its transformation to crystalline minerals during pedogenesis.

Successional patterns of BNF and denitrification

The overall pattern of biological nitrogen fixation that characterised the Llaima volcano chronosequence supports a general model of succession that can be described as having a distinctive progressive phase, with a marked peak of BNF in mid-succession (ecosystem stage 3), followed by sharp declines at about 3500 years of soil development in all N-fixing community types (Fig. 4). Such a pattern is consistent with the hypothesis of an early retrogressive phase of ecosystem development on soils of Llaima volcano, beginning at 3500 years. An initial progressive phase of ecosystem development follows a similar increasing trajectory for the rates of BNF and the contents of organic C and P in soils, with peaks at an intermediate successional stage. The hypothetical retrogressive phase of ecosystem development in the Llaima chronosequences is marked by pronounced declines in total soil C and P and BNF in soils older than 3500 years.

The similar trajectories of BNF by the different N-fixing community types (Fig. 4b) along the chronosequence of the Llaima volcano support the hypothesis that common factors become limiting for BNF as succession progresses, such as the observed decline in total soil C and P. In addition, we observed that N fixation rates estimated for foliose lichen epiphytes were increased by about two orders of magnitude after water addition to samples incubated in the laboratory. This observation led us to conclude that water may also be a major limiting factor for BNF by forest epiphytes, especially during seasonal droughts, a limitation that possibly will become more critical, according to current predictions of global trends in climate for the southern temperate region (Diffenbaugh & Field 2013). The same argument could apply to the contribution to BNF of the litter layer, as acetylene reduction activity was nearly undetectable during dry spells in the field.

Estimates of overall inputs from heterotrophic N fixation (soil plus litter) in the Llaima volcano chronosequence for the peak of the progressive phase (about 800 years) are in the order of 3.0 kg N ha^{-1}year^{-1}, fitting well to known values of non-symbiotic N fixation reported for other temperate forests in the Northern and Southern hemispheres (0.01–12 kg N ha^{-1}year^{-1}; reviewed by Reed et al. 2011). Forest litter alone showed maximum rates of heterotrophic N fixation of 0.97 kg N ha^{-1}year^{-1}, much lower than maximum rates reported (Crews et al. 1995) for the litter layer during the early phases of a Hawaiian forest chronosequence (1.25 kg N ha^{-1}year^{-1}). In the Hawaiian volcanoes the content of lithophilic elements, including sources of P such as slowly soluble calcium phosphates, is at its maximum during the early phases of succession, when available N is at its minimum level, which may explain the high rates of non-symbiotic N fixation recorded in early succession (Crews et al. 1995, 2000).

Successional patterns documented for this volcanic chronosequence in south-central Chile differ greatly from the patterns observed in post-glacial chronosequences in

New Zealand. Despite pronounced declines in all forms of P with stand age (Parfitt et al. 2005), heterotrophic N fixation did not drop significantly in the New Zealand chronosequences (Menge & Hedin 2009), even if a retrogressive phase is recognised. This contrasting successional pattern in BNF is probably due to the fact that C contents did not decline in advanced stages of succession in New Zealand (Richardson et al. 2004), providing a source of energy for heterotrophic diazotrophs.

Estimates of symbiotic-N-fixation inputs by foliose lichen epiphytes in the Llaima volcano chronosequence resemble values measured in New Zealand temperate rainforests (2 kg N ha^{-1}year^{-1}; Menge & Hedin 2009). Symbiotic N fixation follows the same pattern with successional age in both systems, reaching peak values at intermediate stages of ecosystem development (Fig. 4). In our study area, field rates of N fixation during rainy days in spring and summer will likely be similar to the ARA estimates from laboratory incubations. It can be predicted that rain pulses will stimulate episodic increases of N fixation by epiphytes in Chilean forests, in contrast to less variable rates in moister New Zealand rainforests. Although their contribution to N fixation was not evaluated in the present study, the presence of actinorhizal shrubs, such as *Discaria* and *Colletia*, in early- and late-successional stages in Chilean post-volcanic succession can potentially elevate estimates of N inputs via BNF during the early and late phases of ecosystem development.

Denitrification showed trends that were opposite to those of BNF across the chronosequence of sites in Llaima volcano (Fig. 4). The successional stage that had the highest overall rate of N fixation (stage 3; 750 years of substrate age) also presented very low rates of gaseous N losses through denitrification. In accordance with our expectations, losses of biologically available forms of N due to denitrification processes in the forest soil tend to decline through the progressive and maximum phases of ecosystem development on the Llaima volcano. Losses of N increased again in the stands with higher substrate ages, leading to the establishment of conditions that bring up the retrogressive phase.

Balance of gaseous N inputs and outputs and δ^{15}N patterns

Our simple calculations of gaseous N exchanges during the initial stages of ecosystem development on the Llaima volcano, based on laboratory incubations, suggest that inputs of N through symbiotic N fixation by *Racomitrium* moss on denuded soils of recent lava flows are not sufficiently high to compensate for the substantial ecosystem N losses through denitrification in the same soils. This negative balance of ecosystem gaseous N inputs and outputs for the initial successional stage strongly suggests a lack of N retention in young soils from the Llaima volcano chronosequence. In contrast, during the progressive and maximum phases of ecosystem development in the Llaima volcano chronosequence, the rates of symbiotic N fixation by foliose lichen epiphytes, as well as by heterotrophic N fixers in the litter and mineral soil layers, tend to compensate and exceed basal denitrification rates, leading to substantial N accumulation in organic soil and biomass, with a positive budget of gaseous forms of N. Gaseous N inputs and outputs become balanced again in the advanced retrogressive stages in the chronosequence.

The pattern of increase in ^{15}N isotopic signal towards the older stages in the Llaima volcano chronosequence, after reaching its lowest value in mid-succession (Fig. 5), is consistent with trends reported for other temperate forest

chronosequences with a retrogressive phase. One of the mechanisms that explain this late-successional enrichment in the heavier N isotope is the increment of mycorrhizal associations as succession progresses and nutrient limitation in soils becomes intensified. A second mechanism that could enhance δ^{15}N in soils is the observed increase in gaseous N loss through denitrification during ecosystem development (Hobbie & Högberg 2012). Results of the present work give support to the second mechanism explaining the ^{15}N pattern along the Llaima chronosequence.

The data from the Llaima volcano chronosequence reveal that changes in the partial ecosystem N budget based on gaseous N inputs and outputs (Fig. 4d) follow approximately the opposite pattern of the natural abundance of ^{15}N in forest soils (Fig. 5). During the progressive phase of succession, a depleted ^{15}N isotopic signal was associated with decreasing N losses via denitrification, to reach its lowest level in mid-succession. The hypothetical retrogressive phase can then be recognised by the significant ^{15}N isotopic enrichment in millennial-old soils, which reflects enhanced N losses via denitrification and possibly organic N losses to stream waters (Houlton et al. 2006). High gaseous N losses are associated with the retrogressive phase of succession, which occurs in the oldest substrates in the Hawaiian forest ecosystem chronosequences (Hedin et al. 2003).

Conclusions

Our main conclusion is that the pattern of BNF during ecosystem development on volcanic soils of the Llaima volcano, south-central Chile, is related to the strong patterns of C and P accumulation in soils up to mid-succession (Fig. 3). Accordingly, the subsequent decline in ecosystem development and sharp decline in BNF (Fig. 4) is related to a pronounced decline in total soil contents of C and P starting at approximately 3500 years of soil age. Moreover, this decline in C and P and drop in BNF is linked to growing denitrification rates. Our results support the prediction that post-volcanic succession in south-central Chile follows the general model of ecosystem development characterised by progressive, maximal, and retrogressive phases, as described for temperate and tropical long-term chronosequences (Peltzer et al. 2010). In support of the hypothesis, we provide evidence that N does not accumulate indefinitely in soil and biomass during ecosystem development. Old-growth stages in the Llaima volcano chronosequence appear to be at steady state with respect to gaseous N inputs and outputs, as BNF equilibrates denitrification outputs. During the hypothetical retrogressive phase, N should become limiting for ecosystem productivity because hydrologic losses of recalcitrant forms of N from soil humus (Perakis & Hedin 2002) largely surpass BNF rates. Such a condition of strong N limitation has been documented for old-growth *Nothofagus*-dominated forests occurring on Andisols in the southern Chilean and Argentinean Andes, where the main form of N loss in forest streams is dissolved organic N (Perakis & Hedin 2002). Although in the case of the Llaima volcano chronosequence, we lack data for non-biologically controlled, potential hydrologic outputs of organic nitrogen to streams or N inputs in precipitation, we assume that these vectors would be similar to values measured in other forested areas from south-central Chile (Perakis & Hedin 2002). Estimates of hydrologic vectors for the Llaima volcano forests would allow a more complete account of the

ecosystem N budget, thus providing a better model for the mechanisms of N limitation during the retrogressive phase.

The retrogressive phase in the Llaima chronosequence may begin after 3500 years of substrate age, which is considered to be mid-succession. This early onset of retrogression is in contrast with studies that report biogeochemical evidence for retrogression at a much later substrate age. In the Franz Josef Glacier (New Zealand) and Hawaiian volcanic chronosequences, the retrogressive phase begins at 60 000 and 1.4 million years of substrate age, respectively. The time at which retrogression may begin in different long-term chronosequences varies widely across biomes depending on regional climate and geologic substrate types (Peltzer et al. 2010). In volcanic soils, the rates of change from non-crystalline precursors to crystalline minerals are more rapid under warmer/drier climates (Ugolini & Dahlgren 2002). Accordingly, the capacity of C and P occlusion would be lost at more rapid rates in this type of climate, and hence retrogression could begin earlier in the Llaima volcano chronosequence, strongly influenced by its seasonal Mediterranean climate, with characteristically warm and dry summers. A chronosequence study on volcanic soils in a mountain region of Mexico, under strongly seasonal climate (Peña-Ramírez et al. 2009), shows similar biogeochemical patterns, with soil C storage decreasing by 30% between 10 000 and 35 000 years of soil age associated with the reduction of the Al-organo-mineral soil complexes. We propose that sharp declines in soil C mark the onset of the retrogressive phase of chronosequences on volcanic soils, especially because of the transformation of non-crystalline to crystalline soil minerals during pedogenesis, which should also lead to a concomitant decline in total and available soil P.

Acknowledgements

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Appendix 1. Field water content (% wet weight) of samples of three different N-fixing community types: foliose lichen epiphytes, litter samples and surface soil (first 10 cm), measured in the spring of 2012 for different ecosystem stages (1 to 7, see Table 1) in the long-term chronosequence of the Llaima volcano, Conguillío National Park, Chile. Values are means ± 1 SE (n = 7 samples per site).

Chronosequence stage	Epiphytes	Litter	Soil
1	10.3 ± 0.7	11.6 ± 1.0	6.4 ± 0.6
2	15.1 ± 1.3	19.4 ± 0.8	30.1 ± 3.9
3	11.1 ± 0.6	11.7 ± 2.8	23.3 ± 6.5
4	18.1 ± 1.8	44.3 ± 3.7	45.1 ± 5.9
5	19.7 ± 5.2	48.0 ± 3.8	39.8 ± 2.8
6	12.3 ± 1.0	23.3 ± 4.0	24.5 ± 2.3
7	11.4 ± 0.6	22.15 ± 3.5	37.7 ± 1.1

Appendix 2. Acetylene reduction activity rates (nmol ethylene kg $DW^{-1}day^{-1}$) estimated in the laboratory and in the field measured in the spring of 2012 for three N-fixing community types: foliose lichen epiphytes, litter layer, and surface soil (first 10 cm), at seven ecosystem stages (see Table 1) in the long-term chronosequence of Llaima volcano, Conguillío National Park, Chile. Values are means ± 1 SE (n = 6 per N-fixing community types).

Stage	Laboratory assays			Field assays		
	Epiphytes	Litter	Soil	Epiphytes	Litter	Soil
1	53.7 ± 19.7	12.6 ± 1.5	0.0 ± 0.0	2.9 ± 0.2	0.3± 0.2	0.2 ± 0.01
2	103.8 ± 42.8	6.1 ± 0.9	1.7 ± 0.6	2.3 ± 1.9	2.0 ± 0.4	0.8 ± 0.2
3	818.2 ± 65.0	23.1 ± 2.3	3.1 ± 0.5	0.5 ± 0.8	2.0 ± 2.4	1.8 ± 1.0
4	227.2 ± 55.7	19.5 ± 1.5	2.4 ± 0.3	2.8 ± 3.1	10.6 ± 1.4	2.2 ± 0.6
5	55.7 ± 18.2	12.2 ± 2.3	1.6 ± 0.6	1.2 ± 3.9	9.6 ± 0.3	0.2 ± 0.13
6	690.9 ± 116.5	14.2 ± 1.5	1.1 ± 0.4	0.1 ± 0.24	0.3 ± 2.7	1.9 ± 1.1
7	132.1± 30.5	18.3 ± 2.1	1.6 ± 0.5	0.2 ± 0.0	0.0 ± 0.8	1.3 ± 0.3