SHORT COMMUNICATION

Physiologia Plantarum

Evidence for the translocation of fixed N in the N₂-fixing lichen *Stereocaulon vesuvianum*

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Abstract

The fruticose lichen *Stereocaulon vesuvianum* is among the most abundant and widespread lichens in upland Britain. It typically produces cephalodia (nodules) that contain the cyanobacterium *Stigonema*, which can fix atmospheric nitrogen. However, over much of England, Wales, and southern Scotland *S. vesuvianum* no longer produces cephalodia and does not fix nitrogen, a morphological change linked to elevated atmospheric nitrogen deposition. This provided a unique opportunity to compare the ^{15}N natural abundance signatures in N₂-fixing and non-N₂-fixing lichen populations, keeping in mind that fixed nitrogen has a ^{15}N content close to that of atmospheric N₂ while, in comparison, several components of atmospheric combined N (e.g. nitrate and ammonium in precipitation) tend to be ^{15}N depleted. We found that in N₂-fixing samples, there was a steep gradient in ^{15}N relative abundance in the terminal 15 mm of thallus branches (pseudopodetia), changing from ^{15}N depleted tissues at 10–15 mm below the tips to values close to that of atmospheric N₂ at the apices while in non-N₂-fixing samples thallus branches were uniformly ^{15}N depleted. The ^{15}N gradient in N₂-fixing material could not be explained by the presence of cephalodia since these are more abundant towards branch bases. The data provide the first evidence in lichens of translocation of recently fixed N to sink regions of active growth and production of asexual reproductive propagules, bringing lichens into line with N source-sink relationships in N₂-fixing plant symbioses.

1 | INTRODUCTION

Symbiotic associations involving diazotrophic bacteria are the principal agents of nitrogen (N) fixation in many ecosystems (Sprent, 1987; Cleveland et al., 1999; Reed et al., 2011; Davies-Barnard & Friedlingstein, 2020). Such associations achieve high rates of N₂-fixation compared to free-living populations of diazotrophs. In N₂-fixing plant symbioses, the primary sinks for recently fixed N are regions of active vegetative growth and developing reproductive structures. In legumes, these are the shoot apices, young expanding leaves and the developing fruits and seeds (Bray, 1983; Pate, 1989),

and in actinorhizal trees, the developing buds (Baker et al., 1997). In the angiosperm *Gunnera* and the aquatic fern *Azolla*, which host cyanobacterial symbionts, ^{15}N-labelling has again shown that the shoot and thallus apices, respectively, and developing leaves are primary sinks for fixed N (Kaplan & Peters, 1981; Stock & Silvester, 1994) while in the hornwort *Anthoceros*, the sporophytes are strong sinks for N₂ fixed by the symbiotic *Nostoc* (Stewart & Rodgers, 1977). Against this background, it would seem reasonable to predict that in lichens containing cyanobacteria, the growing margins and apices are the main sinks for newly fixed N. However, data pertaining to this question are sparse.

Millbank and Kershaw (1969) and Kershaw and Millbank (1970) quantified the migration of ^{15}N-labelled products of N$_2$-fixation from *Nostoc* in cephalodia (N$_2$-fixing nodules) of *Peltigera* 'aphthosa' (= *P. britannica*) into the adjacent mycobiont. However, they did not identify specific N sinks. Evidence for translocation of N in lichen thalli unrelated to N$_2$-fixation has come from Ellis et al. (2005) and Kytöviita and Crittenden (2007), who demonstrated that ^{15}N-labeled NH$_4^+$, NO$_3^-$ and glycine introduced into the lower horizontal strata of mat-forming lichens (*Cladonia portentosa*, *C. stellaris* and *Stereocaulon paschale*) migrated upwards towards, and into, new apical growth over distances of 20–80 mm during periods of 2–24 months; the authors also provided evidence that the observed translocation was physiologically dependent.

Here we present ^{15}N natural abundance data for *Stereocaulon vesuvianum* Pers. This is a montane tripartite lichen with a circumboreal/circumarctic distribution and is arguably the most widespread and abundant fruticose species in upland Britain. It produces cushion-like thalli comprised of many to numerous erect pseudopodetia (thallus branches) that attach firmly to rock surfaces at their bases (Figure 1a). *Stereocaulon vesuvianum* typically develops cephalodia (Figure 1b & bii) containing the cyanobacterium *Stigonema* (Lavoie et al., 2020) that fix atmospheric N$_2$ (Crittenden, 1975). However, in regions of Great Britain with elevated rates of atmospheric N deposition (> *c.* 10 kg N ha^{-1} y^{-1}) cephalodium development in *S. vesuvianum* is either infrequent or absent, while in other respects, the general habit of the thallus is similar at background and N-enriched sites (Crittenden et al., 2023). Since combined inorganic N in atmospheric deposition is predominantly ^{15}N-depleted compared to atmospheric N$_2$ (see Crittenden et al. (2023) for discussion), we hypothesised that loss of N$_2$-fixing capacity in *S. vesuvianum* would change the ^{15}N thallus signature. Indeed, differences in such signatures between N$_2$-fixing and non-fixing samples provided evidence of translocation of recently fixed N.

2 | MATERIALS AND METHODS

Cephalodiate (N$_2$-fixing) and acephalodiate (non-N$_2$-fixing) material of *Stereocaulon vesuvianum* was collected in September 2016 from three sites in Great Britain experiencing contrasting annual rates of N deposition (Table 1). Mean annual total inorganic N deposition values at these locations for the period 2014–16 were provided by CEH Edinburgh from 5 km × 5 km gridded fields of modelled N deposition values and ranged between 6–40 kg N ha^{-1} yr.$^{-1}$ (Table 1). Note that Hogan et al. (2010) have earlier shown that modelled values of N deposition are as strong as, or stronger than, measured values as predictors of lichen chemistry and physiology. At each of the three sites, replicate thalli of *S. vesuvianum* were collected at ten spots ≥10 m apart (*n* = 10). Thalli were removed from rock surfaces with a scalpel, placed in a Ziplock polythene bag (sufficient material to ±half fill a 1 litre capacity bag) for transport and laid out to air dry at room temperature within 24 h of collection. Air-dried material was stored at −15°C. Powder-free latex gloves were worn when handling lichen material both in the field and in the laboratory.

Air-dried thalli in each replicate collection were separated into pseudopodetia, which were meticulously cleaned of adhering plant,
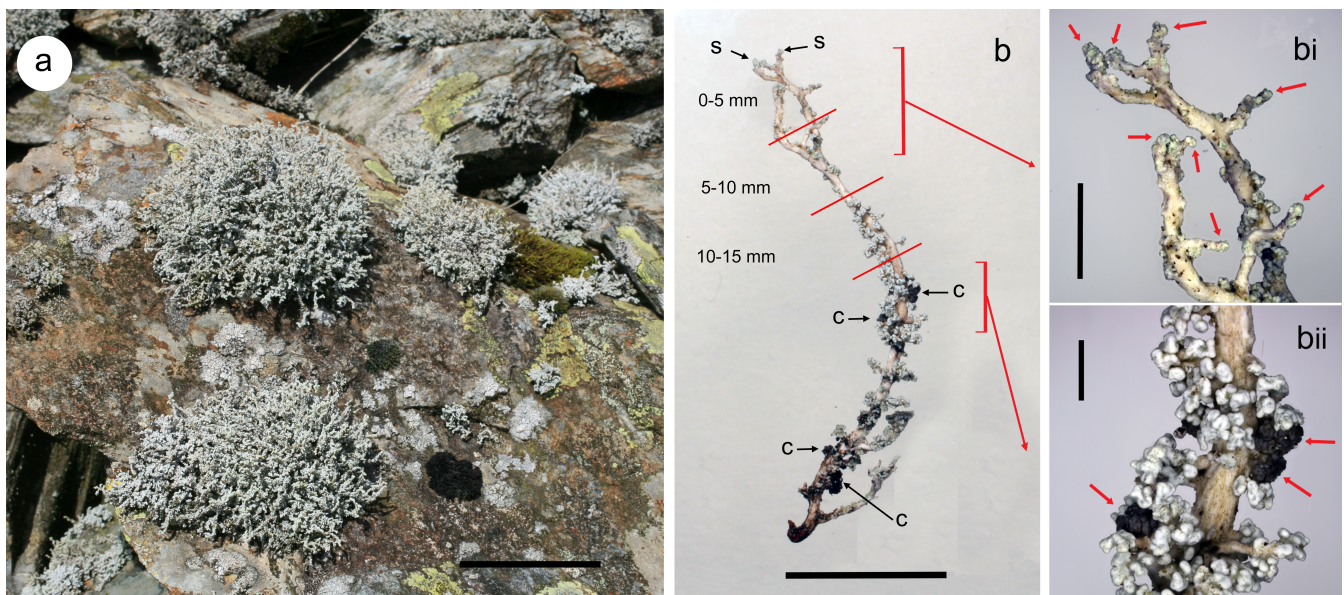

FIGURE 1 Habit and morphology of *Stereocaulon vesuvianum* together with an indication of the thallus segments analysed in this study. a, Thalli of *S. vesuvianum* growing on slate mine spoil at Site 2 (Gwynedd, Moderate N deposition) each comprising numerous aerial pseudopodetia (branches); b, a single pseudopodetium from a thallus collected at Site 1 (Highland, Low N deposition) showing the three 5 mm long segments analysed in the present work (c = cephalodia (N$_2$-fixing nodules), s = soralia (sites of asexual diaspore production)); bi, detail of apical region with soralia (arrows); bii, detail of lower region with dark cephalodia (arrows) surrounded by abundant phyllocladia (photosynthetic squamules/granules). Scales: a = 5 cm; b = 10 mm; bi = 2 mm; bii = 1 mm. See Table 1 for further site details.

TABLE 1 Details of collection sites in Great Britain for *Stereocaulon vesuvianum* Pers. together with modelled mean annual total inorganic N deposition values for 2014–16. Specimens analysed from Site 2 (Moderate N deposition) did not have cephalodia.

Site descriptor	Site location	Nat. Grid Ref.	Altitude (m)	Total N dep (kg ha^{-1} yr.$^{-1}$)	Cephalodium abundance
1. Low N deposition	Coir' a' Ghiubhsachain, Ullapool, Highland	NH 0984	200–300	6.4	Abundant
2. Moderate N deposition	Cwm Trwsgl, Beddgelert, Gwynedd	SH 5449	300–400	11.0	Rare
3. High N deposition	Wythburn Fells, Grasmere, Cumbria	NY 3012	450–650	40.3	Absent

animal and mineral contamination and then cut into 5 mm long segments from the apices downwards: 0–5 mm, 5–10 mm and 10–15 mm (Figure 1b); regions of peudopodetia further from the apices were considered too heavily contaminated with the above to include in analyses. Segments from 15–25 pseudopodetia in each replicate collection were combined, then oven-dried at 40°C, reduced to powder in a ball mill, re-dried and then the powder weighed into ultra-clean tin capsules (Elemental Microanalysis Ltd). Total thallus N and ^{15}N:^{14}N ratios were determined using a CN analyser coupled to an isotope ratio mass spectrometer. ^{15}N natural abundance is expressed in δ notation (‰): $\delta^{15}N = ((R_{sample}/R_{standard})-1) \times 1000$, where R is the ^{15}N:^{14}N ratio of the sample and the international standard (AIR-N2). Further details of collection sites and methods are provided in Crittenden et al. (2023).

The distribution of cephalodia along the length of pseudopodetia was quantified in 100 samples from Site 1 (Low N deposition), ten from each of ten spots as above. The presence or absence of cephalodia was recorded for segments 0–5 mm, 5–10 mm, 10–15 mm and below 15 mm measured downwards from the pseudopodetium tip. Note that counting cephalodia is problematic owing to, amongst other things, coalescence of enlarging cephalodia.

The statistical significance of within-site variation among segments in δ^{15}N and %N values was tested by one-way ANOVA followed by the Holm-Sidak multiple comparison procedure performed in SigmaPlot 11 (Systat Software Inc.). Data were checked for normality and homogeneity of variances and, where test assumptions were not met, data were either log- or square root-transformed.

3 | RESULTS AND DISCUSSION

In N$_2$-fixing material of *Stereocaulon vesuvianum* there was a steep gradient in δ^{15}N values along the uppermost 15 mm length of pseudopodetia (Figure 2a). Values were slightly positive in the apical segment and became increasingly negative with depth. By contrast, in non-N$_2$-fixing specimens, δ^{15}N values were ±constant along the 15 mm length of pseudopodetia analysed (Figure 2b & c). Thus, it would appear that the process (−es) generating the δ^{15}N gradient in *S. vesuvianum* from the low N deposition site did not operate in material exposed to elevated N deposition.

At first sight, the presence or absence of N$_2$-fixation is the obvious factor determining the contrasting patterns of δ^{15}N in *S. vesuvianum*. According to Zhang et al. (2014) ammonium produced by nitrogenase has a δ^{15}N value of −1 ‰ and excised cephalodia of

S. vesuvianum from the low N deposition site had a value of 0.76 ± 0.18 ‰ ($n = 10$, ± 1 SE) (Crittenden et al., 2023), a figure similar to that measured here in the apical segment of 0.80 ± 0.85 ‰ ($n = 10$, ± 1 SE). By contrast, most inorganic N components of atmospheric deposition are δ^{15}N negative (see Crittenden et al. (2023) for discussion). However, the comparatively high δ^{15}N values in the pseudopodetium apices cannot be explained by the presence of cephalodia since in British material, and as reported by Galloway (1985) in New Zealand specimens, these are more abundant in lower, older regions of thalli and are infrequent at the apices (Figure 1b; but see Stenroos et al. (2016)). This qualitative observation was supported by counts of presence/absence of cephalodia with increasing distance from the pseudopodetium tip (Figure 3), revealing a distribution that is the inverse of the δ^{15}N gradient suggesting that the sink strength for newly fixed N at the apices is strong. Note also that cephalodia increase in size and number and often coalesce with increasing distance/age. It is probable, therefore, that the apices are primary sinks for recently fixed N, which is translocated upwards from lower cephalodiate regions; the apices of pseudopodetia are assumed to be the zones of new growth, and they also frequently produce soralia (asexual reproductive structures; Figure 1b & bi). Rates of N$_2$-fixation in *S. vesuvianum* are low compared to many other lichens containing cyanobacteria (Crittenden et al., 2023). Accordingly, uptake of combined N from atmospheric deposition is likely to represent a large proportion of total N intake even in N$_2$-fixing material in which it could be an important N source for continuing thallus development in lower segments of pseudopodetia if fixed N is primarily translocated to apical sinks. The negative δ^{15}N values in non-N$_2$-fixing *S. vesuvianum* are consistent with the capture of ^{15}N-depleted atmospheric N deposition.

Nonetheless, gradients in thallus δ^{15}N values can also develop in non-N$_2$-fixing lichens. Ellis et al. (2003) examined variation in ^{15}N natural abundance in eight species of non-N$_2$-fixing mat-forming lichen from geographically disparate heathland or peatland sites. The term 'mat-forming' refers to terricolous lichens that grow vertically upwards, often forming closed canopies, and that undergo large-scale senescence in the basal regions of the thalli (Crittenden, 1991). Ellis et al. (2003) found a repeating general pattern, independent of contact with underlying soil, of maximum values of ^{15}N natural abundance in the thallus apices declining to a minimum value at c. 30 mm downwards from the mat surface. Citing findings by Taylor et al. (1997) on elevated δ^{15}N values in basidiocarps, the authors suggested that upward translocation of ^{15}N-enriched cytosolic N (e.g. protein) from senescing basal strata of thalli (i.e. internal recycling) in favour of recalcitrant ^{15}N-depleted structural N (e.g. chitin) might contribute to
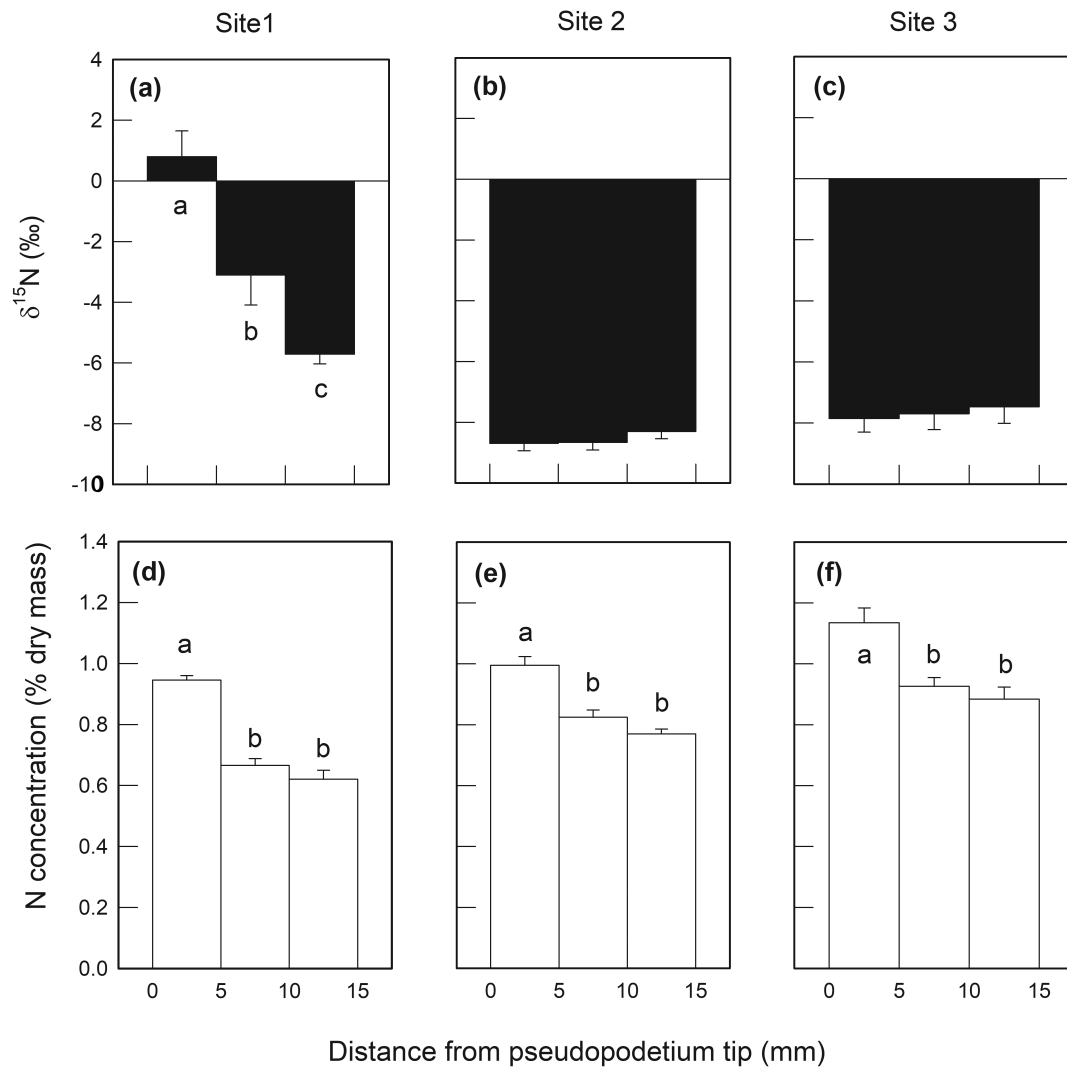
FIGURE 2 Relationships in *Stereocaulon vesuvianum* between distance from the pseudopodetium tip and (a-c) $\delta^{15}N$ value and (d-f) total thallus N concentration. Results are shown for *S. vesuvianum* collected from three locations: Highland (Site 1, Low N deposition, a & d), Gwynedd (Site 2, Moderate N deposition, b & e) and Cumbria (Site 3, High N deposition, c & f) (see Table 1). Mean values are plotted ($n = 10$) ± 1 SEM, and within each site values assigned the same letter are not significantly different at the $p \leq 0.05$ level (one-way ANOVA and the Holm-Sedak *post hoc* test). Raw data are available in Table S1.

the observed variation in ^{15}N natural abundance with depth in lichen mats. A corollary to this proposal is that in non-mat-forming species that do not undergo large-scale senescence, such gradients will be weaker or absent. This finds support in the present results for *S. vesuvianum* in which pseudopodetia are firmly attached to rock substrata and do not undergo large-scale senescence and in which $\delta^{15}N$ values are uniform along the length of acephalodiate pseudopodetia.

Notwithstanding the above explanations, it remains possible that internal recycling of N in pseudopodetia of *S. vesuvianum* might have at least contributed to the $\delta^{15}N$ gradient in cephalodiate material. Recovery and upward migration of ^{15}N enriched cytosolic N from, for example, senescing phyllocladia (squamules containing the green algal symbiont) in older basal zones of pseudopodetia in favour of ^{15}N-depleted structural N (cf. Taylor et al., 1997) might occur at the low

N-deposition site while at Site 3 the exceptionally high rate of modelled N input is likely to N-saturate thalli potentially weakening or eliminating the N sink strength at the apices and inhibiting upward migration of N. However, although such inhibition is plausible at Site 3 it is less so at Site 2 where the moderate rate of N deposition (11 kg N ha^{-1} yr.$^{-1}$) is only marginally greater than the putative critical load for cephalodium development (c. 10 kg N ha^{-1} yr.$^{-1}$) and where cephalodia do indeed occasionally develop (Crittenden et al., 2023). At this location, a vertical gradient in $\delta^{15}N$ values would probably be evident in pseudopodetia if processes other than N_2-fixation drive its development. Note that Ellis et al. (2003) report that in the mat-forming lichen *Cladonia portentosa*, the vertical gradient in $\delta^{15}N$ values is not modified at N-enriched sites. Accordingly, translocation of recently fixed N remains the most credible explanation for the $\delta^{15}N$ gradient reported in Figure 2a.
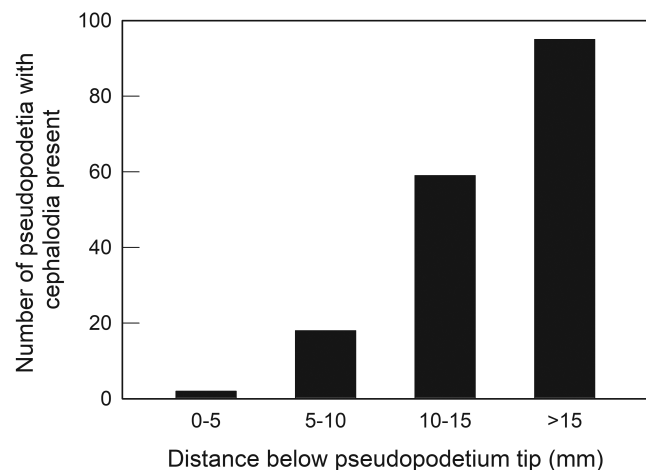
FIGURE 3   Relationship in *Stereocaulon vesuvianum* between distance from the pseudopodetium tip and the presence or absence of cephalodia. Data are for 100 pseudopodetia from the Low N deposition site (Site 1, Table 1). Raw data are available in Table S1.

The pattern of thallus N concentration values among the three segments was similar in both N_2-fixing and non-N_2-fixing material (Figure 2d–f). Although in this case, thallus N concentrations were higher in non-N_2-fixing samples from N-polluted sites than in the N_2-fixing samples from a background site, when data for many sites were considered, there was little evidence that *S. vesuvianum* accumulated N by luxury consumption (Crittenden et al., 2023). The higher N concentrations in young apical tissue are consistent with data for other lichen species (e.g. Crittenden, 1991; Ellis et al., 2003) and might reflect e.g. thinner fungal cell walls and a higher protoplast:cell wall ratio, and higher enzyme concentrations per unit mass in apical tissues.

The availability of both N_2-fixing and non-N_2-fixing populations of *S. vesuvianum* provides a unique opportunity to examine links between diazotrophy and lichen chemistry. Data presented here are consistent with the migration of recently fixed N into apical tissues of pseudopodetia in line with known sinks for fixed N in other N_2-fixing photoautotrophic symbioses.

AUTHOR CONTRIBUTIONS

PDC designed the research, obtained the funding, undertook the field and preparative laboratory work, interpreted the results and wrote the paper. BT made the total N and $\delta^{15}N$ measurements.

CONFLICT OF INTEREST STATEMENT

There are no competing interests.

DATA AVAILABILITY STATEMENT

The raw data summarized in Figures 2 and 3 are available online in the Supporting Information section at the end of the article.

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REFERENCES

Baker, A., Hill, G.F. & Parsons, R. (1997) Evidence for N feedback regulation of N_2 fixation in *Alnus glutinosa* L. *Journal of Experimental Botany*, 48(306), 67–73. https://www.jstor.org/stable/23695559

Bray, C.M. (1983) *Nitrogen Metabolism in Plants*. Longman, London, UK.

Cleveland, C.C., Townsend, A.R., Schimel, D.S., Fisher, H., Howarth, R.W., Hedin, L.O., Perakis, S.S., Latty, E.F., Von Fischer, J.C., Elseroad, A. & Wasson, M.F. (1999) Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. *Global Biogeochemical Cycles*, 13(2), 623–645. https://doi.org/10.1029/1999GB900014

Crittenden, P.D. (1975) Nitrogen fixation by lichens on glacial drift in Iceland. *New Phytologist*, 74(1), 41–49. https://doi.org/10.1111/j.1469-8137.1975.tb01337.x

Crittenden, P.D. (1991) Ecological significance of necromass production in mat-forming lichens. *Lichenologist*, 23(3), 323–331. https://doi.org/10.1017/S0024282991000464

Crittenden, P.D., Ellis, C.J., Smith, R.I., Wanek, W. & Thornton, B. (2023) Loss of nitrogen fixing capacity in a montane lichen is linked to increased nitrogen deposition. *Journal of Ecology*, 111(2), 280–299. https://doi.org/10.1111/1365-2745.14056

Davies-Barnard, T. & Friedlingstein, P. (2020) The global distribution of biological nitrogen fixation in terrestrial natural ecosystems. *Global Biogeochemical Cycles*, 34(3), e2019GB006387. https://doi.org/10.1029/2019GB006387

Ellis, C.J., Crittenden, P.D., Scrimgeour, C.M. & Ashcroft, C. (2003) The natural abundance of ^{15}N in mat-forming lichens. *Oecologia*, 136(1), 115–123. https://doi.org/10.1007/s00442-003-1201-z

Ellis, C.J., Crittenden, P.D., Scrimgeour, C.M. & Ashcroft, C.J. (2005) Translocation of ^{15}N indicates nitrogen recycling in the mat-forming lichen *Cladonia portentosa*. *New Phytologist*, 168(2), 423–434. https://doi.org/10.1111/j.1469-8137.2005.01524.x

Galloway, D.J. (1985) *Flora of New Zealand: Lichens*. Government Printer, Wellington, New Zealand.

Hogan, E.J., Minnullina, G., Smith, R.I. & Crittenden, P.D. (2010). Effects of nitrogen enrichment on phosphatase activity and nitrogen: phosphorus relationships in *Cladonia portentosa*. *New Phytologist*, 186(4), 911–925. https://doi.org/10.1111/j.1469-8137.2010.03222.x

Kaplan, D. & Peters, G.A. (1981) The *Azolla-Anabaena azollae* relationship. X. $^{15}N_2$ fixation and transport in main stem axes. *New Phytologist*, 89(2), 337–346. https://doi.org/10.1111/j.1469-8137.1981.tb07494.x

Kershaw, K.A. & Millbank, J.W. (1970) Nitrogen metabolism in lichens. II. The partition of cephalodial-fixed nitrogen between the mycobiont and phycobionts of *Peltigera aphthosa*. *New Phytologist*, 69(1), 75–79. https://doi.org/10.1111/j.1469-8137.1970.tb04051.x

Kytöviita, M.-M. & Crittenden, P.D. (2007) Growth and nitrogen relations in the mat-forming lichens *Stereocaulon paschale* and *Cladonia stellaris*. *Annals of Botany*, 100(7), 1537–1545. https://doi.org/10.1093/aob/mcm249

Lavoie, C., Renaudin, M., McMullin, R.T., Gagnon, J., Roy, C., Beaulieu, M.-E., Bellenger, J.P. & Villarreal, J.C. (2020) Extremely low genetic diversity of *Stigonema* associated with *Stereocaulon* in eastern Canada. *Bryologist*, 123(2), 188–203. https://doi.org/10.1639/0007-2745-123.2.188

Millbank, J.W. & Kershaw, K.A. (1969) Nitrogen metabolism in lichens. I. Nitrogen fixation in the cephalodia of *Peltigera aphthosa*. *New Phytologist*, 68(3), 721–729. https://doi.org/10.1111/j.1469-8137.1969.tb06476.x

Pate, J.S. (1989) Synthesis, transport, and utilization of products of symbiotic nitrogen fixation. In: *Plant Nitrogen Metabolism* (Poulton, J.E., Romeo J.T. & Conn E.E. eds), pp. 65–115. Plenum Press, New York, USA.

Reed, S.C., Cleveland, C.C. & Townsend, A.R. (2011) Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annual Review of Ecology, Evolution, and Systematics*, 42, 489–512. https://doi.org/10.1146/annurev-ecolsys-102710-145034

Sprent, J.I. (1987) *The Ecology of the Nitrogen Cycle*. Cambridge University Press: Cambridge, UK.

Stenroos, S., Velmala, S., Pykälä, J. & Ahti, T. (Eds) (2016) *Lichens of Finland*. LUOMUS, University of Helsinki, Helsinki, Finland.

Stewart, W.D.P. & Rodgers, G.A. (1977) The cyanophyte-hepatic symbiosis. II. Nitrogen fixation and the interchange of nitrogen and carbon. *New Phytologist*, 78(2), 459–471. https://doi.org/10.1111/j.1469-8137.1977.tb04852.x

Stock, P.A. & Silvester, W.B. (1994) Phloem transport of recently-fixed nitrogen in the *Gunnera-Nostoc* symbiosis. *New Phytologist*, 126(2), 259–266. https://doi.org/10.1111/j.1469-8137.1994.tb03944.x

Taylor, A.F.S., Högbom, L., Högberg, M., Lyon, A.J.E., Näsholm, T. & Högberg, P. (1997) Natural ^{15}N abundance in fruit bodies of ectomycorrhizal fungi from boreal forests. *New Phytologist*, 136(4), 713–720. https://doi.org/10.1046/j.1469-8137.1997.00788.x

Zhang, X., Sigman, D.M., Morel, F.M.M. & Kraepiel, A.M.L. (2014) Nitrogen isotope fractionation by alternative nitrogenases and past ocean anoxia. *Proceedings of the National Academy of Sciences of the United States of America*, 111(13), 4782–4787. https://doi.org/10.1073/pnas.1402976111

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