RESEARCH ARTICLE

Leveraging natural history collections to understand the impacts of global change

Loss of nitrogen fixing capacity in a montane lichen is linked to increased nitrogen deposition

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Abstract

1. The circumboreal/circumpolar N_2-fixing lichen *Stereocaulon vesuvianum* is among the most widespread and abundant fruticose species in montane Britain but has lost the capacity to fix N_2 over large areas of the country.

2. To investigate whether loss of N_2-fixation in *S. vesuvianum* is linked to increased N deposition, we examined thallus morphology, physiology and chemistry at twelve locations representing an N deposition gradient of 3–40 kg ha^{-1} year^{-1}. Measurements were made in parallel on a non-N_2-fixing reference species (*Parmelia saxatilis*). The presence or absence of cephalodia (N_2-fixing nodules containing the cyanobacterium *Stigonema* sp) was recorded in over 500 herbarium specimens of *S. vesuvianum* dating back to 1820.

3. Cephalodium abundance in *S. vesuvianum*, and ^{15}N concentration in *S. vesuvianum* and *P. saxatilis*, were strongly negatively correlated with N deposition and particularly with dry deposited N; cephalodia do not form at total N deposition rates ≥8–9 kg ha^{-1} year^{-1}. Other morphological oddities in *S. vesuvianum* at N-polluted sites include increased apothecium (fungal reproductive structure) production and green algal biofilm development. Biofilm covered thalli without cephalodia lacked nitrogenase activity and cephalodia at sites where they rarely develop had nitrogenase activities typical for this species. The presence or absence of cephalodia in herbarium specimens of *S. vesuvianum* suggest that the present-day N-deposition linked gradient in N_2-fixing capacity did not exist in the 19th century and largely developed between 1900–1940.

4. *Synthesis.* We provide clear evidence that N_2-fixing capacity in *S. vesuvianum* has been lost in regions subjected to many decades of enhanced atmospheric N deposition. This loss is consistent with established models of diazotrophy, which identify supply of combined N as an inhibitor of N_2-fixation. Progressive depletion of thallus ^{15}N with increasing N deposition is in line with available data indicating that much atmospheric N pollution is ^{15}N-depleted. Rates of nitrogenase activity in *S. vesuvianum* are low compared to other symbiotic systems and perhaps more likely supplanted by elevated N deposition. We suggest that

other ecosystem compartments with low rates of fixation (e.g. soils) might also be susceptible to N pollution and merit investigation.

1 | INTRODUCTION

Global deposition of nitrogen (N) from the atmosphere has increased by a factor of three since 1850 (Holland et al., 1999; Kanakidou et al., 2016; Lamarque et al., 2013). Over Northern Hemisphere temperate regions the increase has been four-fold with maxima of up to 16-fold for some cultivated lands and mixed forests (Holland et al., 1999; cf. Engardt et al., 2017). There has been considerable interest in how N enrichment from atmospheric deposition might have modified the N cycle in specific habitats. Nitrification has been studied intensively since elevated rates of ammonium (NH_4^+) oxidation were found to be indicators of N saturation (Aber et al., 1998; Chiwa et al., 2019; Corre et al., 2003; Dise & Wright, 1995; Fenn et al., 1998; Isobe et al., 2011; Lovett & Goodale, 2011; Matson et al., 2002; Niu et al., 2016; Venterea et al., 2004) that is, the state in which total N output from an ecosystem is equal to or greater than N input (e.g. Aber et al., 1989; Näsholm et al., 1997). Fewer studies have considered the effect of N pollution on rates of nitrogen (N_2-) fixation.

It is axiomatic that the supply of combined N depresses rates of N_2-fixation in both free-living and symbiotic diazotrophs (Anderson, 2011; Dynarski & Houlton, 2018; Liu et al., 2011; Postgate, 1998; Salvagiotti et al., 2008; Zheng, Zhou, et al., 2019) including both free living (Bhaya et al., 2000) and lichenized cyanobacteria (cf. Kytöviita & Crittenden, 1994). The results of recent fertilization experiments undertaken in the context of N pollution effects are largely consistent with this established paradigm; systems studied include soils, and other compartments, of a temperate forest (Zheng, Zhang, et al., 2019), tropical forests (Cusack et al., 2009; Matson et al., 2015; Wang et al., 2018; Wang, Moore, et al., 2019; Zheng et al., 2016; Zheng, Zhang, et al., 2019) and semiarid Mediterranean shrublands (Ochoa-Hueso et al., 2013), and also legumes (García-Palacios et al., 2012; McPhee et al., 2015; Skogen et al., 2011; Suding et al., 2005; Tognetti et al., 2021), and cyanobacterial associations with bryophytes (Gundale et al., 2011, 2013; Kox et al., 2016; Rousk & Michelsen, 2016; Sorensen et al., 2012; Zackrisson et al., 2004). However, beyond these fertilization experiments, there remains scant evidence that capacity for N_2-fixation has declined in natural environments subjected to decades of chronic N pollution.

Ochoa-Hueso et al. (2013) reported a weak negative relationship between N deposition and soil nitrogenase activity across 19 semiarid Mediterranean shrubland sites in Spain exposed to total N deposition rates in the narrow range of 4.3–7.3 kg ha^{-1} year^{-1}. Salemaa et al. (2019) studied N_2-fixation capacity in forest understorey feathermosses along a north to south gradient of increasing N deposition in Finland (total N deposition ranging from 0.9–4.4 kg ha^{-1} year^{-1}): nitrogenase activity was appreciable at northernmost sites and declined to zero at southernmost sites. However, two potential confounding factors might have influenced the results of this latter study. First, stand age of the selected forest sampling sites progressively decreased southwards and feathermoss-associated N_2-fixation has been shown to be positively correlated with stand age linked, in turn, to the inhibitory effects of higher N concentrations in canopy throughfall in younger forests (DeLuca et al., 2007, 2008; Zackrisson et al., 2004). Second, N_2-fixation increased with latitude raising the possibility of a climatic influence. Nevertheless, feathermoss-associated nitrogenase activity also declines near busy roads, but not remote roads, pointing to a possible sensitivity to vehicular N emissions (Ackermann et al., 2012).

Despite the dearth of field survey results to support the hypothesis that anthropogenic N deposition has lowered rates of N_2-fixation, recent efforts to model N cycles have assumed or predicted that such negative feedback occurs in both terrestrial (de Vries et al., 2017; Meyerholt et al., 2016; Wieder et al., 2019) and marine (Jickells et al., 2017; Wang, Moore, et al., 2019; Yang & Gruber, 2016) environments. For example, in their models of European forest growth, de Vries et al. (2017) incorporated negative feedback between N deposition and N_2-fixation based on published results of N-enrichment experiments with feathermosses, and Wang, Moore, et al. (2019) identified a 10% reduction in modelled global marine N_2-fixation between 1850 and 2000 based on estimated changes in nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentrations in ocean water. Moreover, several authors have suggested that quantitative information on ecosystem level rates of N_2-fixation and their response to N pollution remain among the largest uncertainties in the quantification of N cycles (Fleischer et al., 2015; Meyerholt et al., 2016; Wang, Moore, et al., 2019; Wieder et al., 2015).

Lichens typically grow in habitats with low availabilities of N and phosphorus (P) (Crittenden, 1989; Crittenden et al., 1994). They acquire these nutrients principally from atmospheric deposits by efficiently scavenging inorganic forms N and P from solutes in precipitation (Crittenden, 1989, 1998) via high affinity transporter systems (Farrar, 1976; Hogan, 2009). Lichens are widely regarded as being among the most sensitive receivers of atmospheric pollutants in terrestrial ecosystems and their responses to N enrichment are chemical, e.g. accumulation of thallus N (Hyvärinen & Crittenden, 1998), physiological, e.g. up-regulation of surface phosphatase activity

(Hogan et al., 2010), and phytosociological whereby species rich, oligotrophic epiphytic lichen communities are replaced by increasingly eutrophication-tolerant, species poor, assemblages. The changes in epiphytic lichen community composition in response to N deposition are predictable and have proved of considerable value as biological indicators of N pollution (e.g. Geiser & Neitlich, 2007). Lichens containing cyanobacteria, and that fix N_2, are among the most N-sensitive epiphytic species (Geiser et al., 2019). However, there is no evidence that this sensitivity is a direct result of inhibition of N_2-fixation. Further, until now there is little convincing evidence that N_2-fixation is impaired in cyanobacterium-containing lichens where they are still present in areas of elevated N deposition.

During field work in North Wales (Llyn Llagi, SH 647481) in 2006 we encountered large populations of the fruticose lichen *Stereocaulon vesuvianum* Pers. This is a tripartite saxicolous species with a circumboreal/circumpolar distribution. It typically produces cephalodia (= nodules) containing the N_2-fixing heterocystous cyanobacterium *Stigonema* sp. and is arguably one of the most widespread and abundant fruticose lichens in tree-less montane areas of Britain (Figure 1a) where it intercepts atmospheric deposits directly without modification by plant canopies. The North Wales population had morphological oddities including a lack of cephalodia and a pronounced algae-rich biofilm covering the thallus branches (pseudopodetia). Subsequent casual observations indicated that this morphology was typical of *S. vesuvianum* in much of England and Wales in contrast to populations in northern Scotland that are abundantly cephalodiate (and hence N_2-fixing) and largely lack well-developed

algal biofilms. The cephalodiate state is also typical of the species throughout its range in other regions of the world (Lamb, 1977, 1978). These observations led to the present study in which we tested the hypothesis that the observed loss of N_2-fixing capacity in *S. vesuvianum* in Great Britain is linked to anthropogenic N enrichment consistent with the classic model of diazotrophy and recent experimental evidence outlined above.

2 | MATERIALS AND METHODS

2.1 | Lichen collection sites and environmental data

The N_2-fixing lichen *Stereocaulon vesuvianum* and the non-N_2-fixing *Parmelia saxatilis* (L.) Ach. were collected from rock surfaces at ten upland or otherwise treeless sites in Great Britain representing a N deposition gradient with modelled annual total inorganic N deposition ranging from 3–40 kg ha^{-1} year^{-1} (Figure 1b,c, Table 1). *Parmelia saxatilis* was included in the study as a non-N_2-fixing reference species. Earlier collections of *S. vesuvianum* from two additional sites, and made using the same protocol as below, were also included in the study (Table 1). Lichen collections at each site were confined to within a single 5×5 km grid square. Note that *S. vesuvianum* is a very variable species and several varieties have been described. Collections in the present work included both var. *vesuvianum* and var. *nodulosum*. However, Phinney (2016) could find no molecular
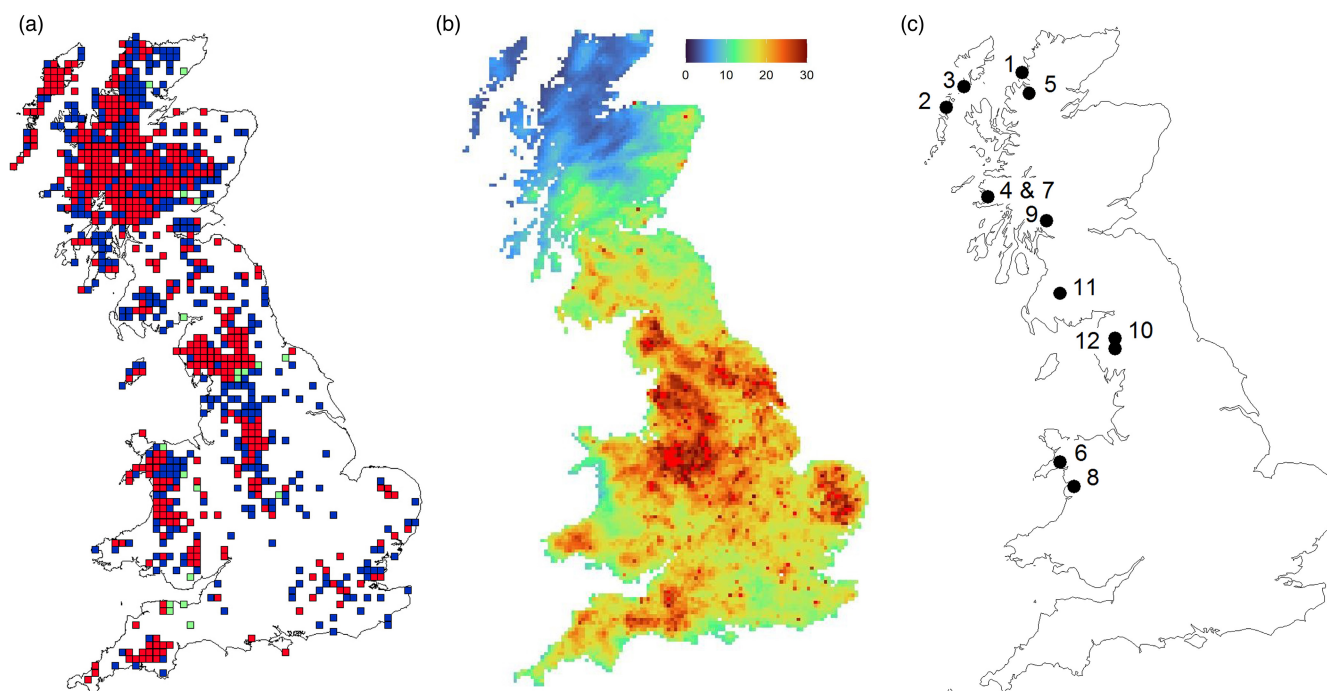

FIGURE 1 Maps of Great Britain showing (a) the distribution of *Stereocaulon vesuvianum* (map courtesy of The British Lichen Society), (b) total N deposition (kg N ha^{-1} year^{-1}) for the period 2014–2016 (map courtesy of CEH Edinburgh) and (c) the location of sampling sites reported in Table 1. Colour coding in (a) refers to dates of records: red = 2000–2021; blue = 1960–1999; green = pre-1960. Units in (b) = kg N ha^{-1} year^{-1}

TABLE 1 Details of collection sites in Great Britain for *Stereocaulon vesuvianum* Pers. and *Parmelia saxatilis* (L.) Ach. together with modelled mean annual deposition values[a] for inorganic nitrogen (N), acidity (H^+) (kg ha^{-1} year^{-1}), and rainfall (mm) (see Figure 1c)

Site No	Site name and location[b]	Nat. Grid ref.	Altitude (m)	Latitude (°N)	Tot wet N	Wet NH_4^+	Wet NO_3^-	Dry NH_y	Dry NO_x	Wet H^+	Rain	Collection date
1	Altandhu, Ullapool, Highland	NB 9813	80–110	58.1	2.00	1.12	0.88	0.47	0.32	0.09	1234	Sept 2016
2	Eaval, Strumore, Western Isles	NF 8960	50–345	57.5	2.71	1.75	0.96	0.51	0.40	0.10	1478	June 2015
3	Loch Creabhat, Tarbert, Western Isles	NG 1193	40–90	57.8	3.01	1.85	1.18	0.47	0.37	0.14	1734	Nov 2017
4	Cailleach Chrìon, Salen, Argyll & Bute	NM 4443	100–180	56.5	5.03	3.26	1.76	0.81	0.43	0.15	1718	Aug 2012
5	Coir' a' Ghiubhsachain, Ullapool, Highland	NH 0984	200–300	57.8	5.39	2.93	2.46	0.55	0.41	0.28	2361	Sept 2016
6	Cwm Trwsgl, Beddgelert, Gwynedd	SH 5449	300–400	53.0	5.41	3.23	2.18	4.76	0.82	0.12	1496	Sept 2016
7	Beinn na h-Iolaire, Salen, Argyll & Bute	NM 4530	360–500	56.4	6.87	4.47	2.40	1.08	0.49	0.19	2119	June 2017
8	Llyn Gafr, Dolgellau, Gwynedd	SH 7114	400–500	52.7	9.98	6.28	3.50	3.91	0.96	0.15	2035	Sept 2016
9	Sìth Mòr, Luss, Argyll & Bute	NS 3097	450–600	56.1	12.34	7.61	4.73	1.71	0.55	0.23	2148	Oct 2018
10	Blencathra, Keswick, Cumbria	NY 3127	550–850	54.6	17.79	11.43	6.36	6.46	0.81	0.18	1726	Sept 2016
11	Knockower, Carsphairn, Dumfries & Galloway	NX 5194	320–470	55.2	21.61	15.81	5.81	3.03	0.67	0.16	2153	Oct 2017
12	Wythburn Fells, Grasmere, Cumbria	NY 3012	450–650	54.5	35.84	22.66	13.18	3.72	0.83	0.34	3951	Sept 2016

[a]Data for period 2014–16 (see Section 2).

[b]Sites are ranked in order of increasing modelled annual mean wet deposited inorganic N (see Section 2).

support for a distinction between these two varieties. *Parmelia saxatilis* is a species complex of which three species have been found in Scotland (*P. ernstiae*, *P. serrana* and *P. saxatilis s. str.*) that cannot be distinguished using morphological characters (Corsie et al., 2019). However, the available data suggest that *P. saxatilis* growing on rock is predominantly *P. saxatilis s. str.* (Corsie et al., 2019; B.J. Coppins, pers. comm., June 2021).

In addition to the collections of *S. vesuvianum* and *P. saxatilis*, a range of terricolous N_2-fixing lichens was collected for comparative purposes; this included species with different rates of nitrogenase activity (*Lobaria linita* (Schreb.) Hoffm., *Nephroma arcticum* (L.) Torss., *Peltigera aphthosa* (L.) Willd., *P. malacea* (Ach.) Funck, *P. scabrosa* Th. Fr., *Stereocaulon alpinum* Laurer ex Funck and *S. paschale* (L.) Hoffm.). These species were selected because they grow in broadly similar habitats to *S. vesuvianum* and field measurements of nitrogenase activity are available for each. All were collected from subarctic heathlands at or above treeline near the Abisko National Park, Sweden (68.41°N, 18.66°E) with the exception of *Peltigera scabrosa* which was collected near Muonio in northern Finland (67.70°N, 24.15°E) by L. Myllys (Finnish Museum of Natural History, Helsinki, Finland)

and from the Commander Islands, far eastern Russia (55.00°N, 166.50°E) by D.E. Himelbrant & I.S. Stepanchikova (Saint-Petersburg State University, St. Petersburg, Russia). Note that permits or permissions to collect any of the above species were not required.

Modelled values of N deposition representative for the periods 2012–2014 and 2014–2016 (Table 1) were provided by CEH Edinburgh as four components (kg ha^{-1} year^{-1}): oxidized and reduced wet deposited N (NO_3^- and NH_4^+, respectively), and oxidized and reduced dry deposited N (NO_x and NH_y, respectively). Modelled values were derived from annual mean measured concentrations ($=[x]$) of NO_3^- and NH_4^+ in precipitation, and NO_2, HNO_3, NH_3, and aerosol-associated NO_3^- and NH_4^+ in air; all measurements were results from the UK Eutrophying and Acidifying Pollutants (UKEAP) network operated by DEFRA (https://uk-air.defra.gov.uk/networks/network-info?view=ukeap; RoTAP, 2012). 5 km × 5 km gridded concentration fields for the UK were generated by interpolative modelling. Modelled $[NO_3^-]$ and $[NH_4^+]$ values in precipitation were combined with UK Meteorological Office annual precipitation maps to generate wet deposition fields incorporating enhancements to account for the seeder-feeder effect and occult deposition in upland

regions. Modelled values of $[NO_2]$, $[HNO_3]$ and [aerosol-associated NO_3^-] were combined (=$[NO_x]$) and adjusted to account for local source strength for NO_2 (e.g. road traffic emissions). Modelled values of $[NH_3]$ and [aerosol-associated NH_4^+] were combined (= $[NH_y]$) and further modelled to account for local sources (e.g. numbers of farm livestock). Note that measured $[NO_2]$ represented roughly 75%–85% of $[NO_x]$ values in western rural areas during this study, and $[NH_3]$ around 40%–60% of $[NH_y]$ values. Dry deposition of gases and aerosols was then estimated using vegetation-specific deposition velocities (RoTAP, 2012). All deposition values used in the present work were parameterized for treeless moorland terrain (Smith & Fowler, 2001). Initial selection of lichen collection sites was based on modelled values of total wet deposited inorganic N since, of the four deposition components considered here, wet deposited NO_3^- and NH_4^+ are more closely related to measurements.

Averaged values of annual mean temperature were calculated over the reference period 2010–2019 based on the UK Meteorological Office interpolation of instrumental climate records at a 1 km grid-scale (Hollis et al., 2019), which is made publicly available on a per-year basis via the CEDA Archive (https://catalogue.ceda.ac.uk/).

2.2 | Pre-treatment and preparation of lichen samples

At each collection site, 10 replicate samples of the two lichen species were taken in pairs from rock faces and boulders at spots >10 m apart. Samples were removed from substrata with a scalpel, placed in polythene bags for transport, air-dried at room temperature within 24 h of collection and stored at −15°C. Tips of pesudopodetia of *S. vesuvianum* (generally 5–15 mm long) and terminal lobes of *P. saxatilis* (generally 3–8 mm long) were selected for chemical analysis because they are the least contaminated regions of the thallus; a large number of pseudopodetia and thallus lobes (e.g. *c.* 100–200) from each collection were meticulously cleaned of adhering mineral, plant and animal debris from which a subsample (*c.* 30–60) was ground for analysis (see below). Powder-free latex gloves were worn when handling lichens both in the field and subsequently in the laboratory. During the sorting process differences among sites in the morphology of *S. vesuvianum* became apparent and these morphological characters were noted for each replicate collection. The abundance of cephalodia and apothecia in each collection of *S. vesuvianum* was estimated using the following six point scale: 0 = absent, 1 = rare, 2 = occasional, 3 = frequent, 4 = frequent/locally abundant, 5 = abundant.

N_2-fixing lichens from northern Sweden, Finland and Russia were collected and pre-treated as above. Additional collections of *S. vesuvianum* were made from among the 12 British sampling sites for the purpose of performing nitrogenase assays. These samples were air dried, placed in a growth room at 15°C and in an appropriate day/night light cycle, and assayed within 7 days of collection.

Voucher specimens of *S. vesuvianum* from sampling sites 1 to 12 were deposited in the Natural History Museum, London (BM;

barcodes: BM013762332-43), the National Museum Wales, Cardiff (NMW; Accession Numbers NMW C.2022.17.1-12) and Royal Botanic Garden Edinburgh (E; barcodes: E01003338-49).

2.3 | Determination of ^{15}N natural abundance and total N and P concentrations

Lichen samples for isotopic analysis were oven-dried at 40°C, reduced to powder in a ball mill, re-dried and aliquots of 1–6 mg weighed into ultra-clean tin capsules (Elemental Microanalysis Ltd.). The N concentrations (% dry mass) and $\delta^{15}N$ values (‰) in the dry powdered lichens were determined using a Flash EA 1112 Series Elemental Analyser connected via a Conflo III to a Delta[Plus] XP isotope ratio mass spectrometer (James Hutton Institute) or to a Delta V Advantage (University of Vienna; all Thermo Fisher Scientific). The $\delta^{15}N_{Air-N2}$ values were normalized to their isotopic scale using international reference materials USGS40 and USGS41a (both L-glutamic acid), as well as IAEA-N-1 and -N-2, and IAEA-NO-3 ((NH_4)$_2SO_4$ and KNO_3 standards). Additionally, USGS40 was used as a reference material for the [N] values measured using the peak area output of the mass spectrometer. Aliquots of these standards were analysed with every batch of 20–40 samples. Long term precisions for quality control standards were: [N] = 1.84 ± 0.06% and $\delta^{15}N$ = 2.01 ± 0.22 ‰ (mean ± SD, n = 20, flour, James Hutton Institute) and 0.5% RSD for [N] and 0.10 ‰ SD for $\delta^{15}N$ (long-term measure for a laboratory mixed standard made of sucrose and proline, University of Vienna). Data processing, including blank correction, was performed using Isodat 2.0 (Thermo Fisher Scientific) and exported into Excel. $\delta^{15}N$ values (‰) were calculated as $((R_{sample}/R_{standard}) - 1) \times 1000$, where R is the $^{15}N{:}^{14}N$ ratio of the sample and the international standard (AIR-N_2).

Initially, all lichen samples were analysed at the James Hutton Institute. It was noted that for repeated measurements on *P. saxatilis*, variation was greater than for *S. vesuvianum* and therefore *P. saxatilis* was subjected to re-analysis at the University of Vienna; this yielded consistent results. There was close agreement between the two laboratories for *S. vesuvianum* $\delta^{15}N$ values; a linear regression of James Hutton values on University of Vienna values for samples from sites 3 and 12 had the following coefficient values: r^2 = 0.98, a = −1.9, b = 0.87 (n = 10).

Powdered lichen samples for total P analysis were oven-dried at 40°C, weighed, digested using the sulphuric acid-hydrogen peroxide procedure (Allen, 1989) and then PO_4^{3-} assayed colorimetrically by the malachite green variant of the methylene blue method (Van Veldhoven & Mannaerts, 1987).

2.3.1 | Measurement of nitrogenase activity

Nitrogenase activity in *S. vesuvianum* was quantified by the acetylene reduction method (Stewart et al., 1967). Air dried lichen samples were placed in a water-saturated atmosphere (over water

in a desiccator) overnight and then sprayed with deionized water. Water-saturated pseudopodetia were incubated in air containing 10% acetylene by volume in 21 ml capacity vials containing a moist 10 mm diam. filter paper and closed with a rubber Suba-Seal (Scientific Laboratory Supplies Ltd). Vials were placed under a fluorescent light bank; temperature and PPFR measured within bottles were in the range 20–24°C and 170–200 μmols m^{-2} s^{-1}, respectively. After 1 h ethylene in the headspace gas was analysed by gas chromatography using a 2.13 m long×2 mm internal diameter column of Porapak N as separator, nitrogen gas as carrier at 30 ml min^{-1} and at 60°C, and FID detection (Pye Unicam 4500 GC). Excised cephalodia were incubated in 1 ml capacity disposable syringes containing 10% acetylene in air. A small droplet of water was introduced into the syringe to maintain a saturated atmosphere. After 1 h, 0.5 ml of headspace gas was injected directly from the syringe onto the GC column. Following the termination of assays, thalli and cephalodia were oven-dried at 80°C and weighed. Assays used commercially available acetylene the ethylene content of which was reduced following Witty (1979). 'No acetylene' and 'no lichen' controls were run in parallel with all assays.

2.4 | Examination of archival material in herbaria

Collections of *S. vesuvianum* were examined in herbaria at BM, NMW, E, Leicestershire County Council Museum Service (LSR), Glasgow Museum Herbarium (GLAM), University of Glasgow Herbarium (GL), the Tullie House Museum, Carlisle (CLE) and the Tasmanian Herbarium, Australia (HO), with the purpose of determining whether this species (i) produced cephalodia during the 19th century in areas of Britain where it does not do so today, and (ii) produces cephalodia in regions to the south of the British Isles. Examination of more recent herbarium acquisitions provided information on the current distribution of cephalodiate and acephalodiate populations.

2.5 | Data analysis

Pearson's product moment correlation, performed in SigmaPlot 11 (Systat Software Inc.), was used to identify pairwise relationships between environmental factors (independent variables) and a range of lichen morphological and chemical traits (dependent variables). Data were checked for normality and homogeneity of variances and where test assumptions were not met data were log- or square root-transformed. Significantly correlated independent and dependent variables were further investigated using regression analysis, though taking into consideration correlations that exist among the independent variables (Tables S1 and S2). Regression was based on multimodel inference (Grueber et al., 2011; Symonds & Moussalli, 2011), which is an exploratory technique that can aid interpretation in the relative importance of correlated independent variables. Full multiple regression models were constructed including the four components of N deposition as independent variables, then with all

possible subsets of the independent variables, performed in the MuMIn package of R (Bartoń, 2019). The full and subset models were then ranked by their scores for a corrected Akaike's information criterion (AICc), using the Akaike weight to calculate probability that any given model is the best approximation of the data. Akaike weights were summed, starting from the model with the lowest AICc, until the summed weights were ≥0.95. This procedure identified the model subset that contained the best approximating model at the 95% confidence level. The importance of independent variables was estimated by summing the Akaike weights for each of the models within which a variable occurs, with values potentially in the range 0 to 1. Where appropriate, ordinary linear regression was used to exemplify the relationship between a dependent variable and the most important independent variable, inferred from the multi-model inference.

3 | RESULTS

3.1 | Morphological variation and nitrogenase activity in *Stereocaulon vesuvianum*

The abundance of cephalodia in *Stereocaulon vesuvianum* varied among the twelve collection sites from abundant (Site 3) to zero (Sites 10 and 12). It was significantly negatively correlated with all components of N deposition (Table 2). However, based on multi-model inference dry deposition of NH_y was clearly the most important explanatory variable with a cumulative Akaike weight (CAW) of 0.95 compared to values for other N deposition components of <0.25 (Figures 2a and 3a). Cephalodial abundance was also positively related to latitude (Table 2). Other morphological changes frequently apparent in material at high N deposition sites include elevated numbers of both apothecia and phyllocladia (squamules containing the green algal symbiont), enlarged and flattened or plate-like phyllocladia, and the presence of a green algal biofilm often coating the entire length of pseudopodetia. Each of these characters can be encountered occasionally at low N deposition sites but are especially conspicuous in highly N-polluted areas. Abundance of apothecia was weakly but significantly positively correlated with several components of N deposition (Table 2); the results of the multi-model analysis suggested that the four components of N deposition contributed roughly equally as explanatory variables (Figures 2b and 3b). Phyllocladium morphology and abundance, and the extent of biofilm development proved difficult to quantify reliably. In other respects the general habit of *S. vesuvianum* was similar at background and N-enriched sites. Note that relationships between N deposition and both thallus morphology and thallus chemistry (presented below) were similar for both the 2012–14 and 2014–16 N deposition data sets (Tables S1 and S2); the similarities were particularly strong for the most significant relationships (Figure S1).

Microscopic examination of biofilms on pseudopodetia from N-enriched sites revealed the dominant elements to be filamentous and unicellular green algae including *Hormidiopsis crenulata* (=

TABLE 2 Bivariate Pearson correlation coefficients (r) between lichen thallus variables (abundance of cephalodia and apothecia, concentrations of N ([N]) and P ([P]), and ^{15}N natural abundance (δ^{15}N)) in *Stereocaulon vesuvianum* (Sv, $n = 12$) and *Parmelia saxatilis* (Ps, $n = 10$), and latitude and components of mean annual N deposition[a]

	Latitude	Sv cephalodia	Sv apothecia	Sv [P]	Sv δ^{15}N	Ps [N]	Ps [N]:[P]	Ps δ^{15}N
Wet NO_3^-	−0.602*	−0.824***	0.645*	0.515	−0.638*	0.589	0.747*	−0.522
Wet NH_4^+	−0.612*	−0.839***	0.631*	0.495	−0.619*	0.587	0.778**	−0.404
Wet $NO_3^- + NH_4^+$	−0.466	−0.742**	0.608*	0.574	−0.458	0.580	0.803**	−0.442
Dry NO_x	−0.968***	−0.887***	0.580*	0.577*	−0.962***	0.814**	0.767**	−0.843**
Dry NH_y	−0.924***	−0.948***	0.642*	0.642*	−0.907***	0.929***	0.660*	−0.662*
Dry $NO_x + NH_y$	−0.871***	−0.880***	0.541	0.717*	−0.859***	0.928***	0.677*	−0.687*
Total oxidized N	−0.643*	−0.85***	0.658*	0.658*	−0.673*	0.628	0.776**	−0.564
Total reduced N	−0.761**	−0.922***	0.682*	0.682*	−0.754**	0.735*	0.829**	−0.512
Total N deposition	−0.730**	−0.906***	0.677*	0.677*	−0.734**	0.694*	0.844**	−0.525
Ratio tot NH_y:tot NO_x	−0.793**	−0.772**	0.467	0.567	−0.689*	0.808**	0.471	−0.339
Ratio dry NH_y:dry NO_x	−0.790**	−0.871***	0.541	0.751**	−0.772**	0.935***	0.610	−0.540
Latitude	—	0.847***	−0.575	−0.542	0.968***	−0.798**	−0.701*	0.793**
Sv cephalodia		—	−0.577*	−0.754**	0.817**	−0.896***	−0.817**	0.681*
Sv [P]			—	—	−0.481	0.816**	0.487	−0.514
Sv δ^{15}N				—		−0.732*	−0.663*	0.831**
Ps [N]:[P]						—	—	−0.645*

Note: for the sake of brevity several variables yielding either no or few significant r values have been omitted; these are Sv [N], Ps [P], Sv [N]:[P], rainfall, acid deposition (H^+) and temperature. The full data set is presented in Table S2 together with covariance between N deposition variables.
[a]Deposition data for period 2014–16 (see Section 2).
*Correlation significant at the $p = 0.05$ level; **Correlation significant at the $p = 0.01$ level; ***Correlation significant at the $p = 0.001$ level.

Klebsormidium crenulatum), *Mesotaenium macrococcum* and unicells close to *Chlorococcum* spp and *Coccomyxa* spp (A. Pentecost, personal communication, August 2017). Cyanobacteria were not observed. *Hormidiopsis crenulata* and *M. macrococcum* are common and widely distributed terrestrial species most notably of acid sandy soils (Lukešová, 2001; Rahmonov & Piątek, 2007; Sparrius et al., 2012).

Pseudopodetia of *S. vesuvianum* from unpolluted sites had readily measurable rates of nitrogenase activity. By contrast, material from N-enriched sites which lacked cephalodia and were coated with well-developed algal biofilms had no detectable activity (Figure 4). Cephalodia from Site 6, where they were rare, had similar activity to those from Site 7, where they were frequent.

Comparison of different species of N_2-fixing lichens revealed a non-significant trend for rising thallus δ^{15}N values with increasing rates of nitrogenase activity (Figure 5); of the species compared, *S. vesuvianum* had the lowest values of both measurements.

3.2 | ^{15}N natural abundance, and thallus total N and P

There were marked differences between *S. vesuvianum* and *P. saxatilis* in the apparent responses of thallus chemistry to increasing N deposition. Total thallus [N] and [N]:[P] mass ratio in *P. saxatilis* were positively

correlated with several components of N deposition (Table 2). Thallus [N] values ranged from 0.98% at Site 5 to 1.57% at Site 10 and thallus [N]:[P] ratio from 7.9 at Site 5 to 12.4 at Site 12. Based on multimodel inference, dry deposition of NH_y was the most important explanatory variable in the case of total thallus [N] (CAW = 0.98; Figures 2f and 6a), with wet deposition of NH_4^+ the most important variable in the case of [N]:[P] mass ratio (CAW = 0.50; Figures 2g and 6b). By contrast, in *S. vesuvianum* these measures, while showing variation along the N deposition gradient, were not significantly related to N deposition. Conversely, δ^{15}N values in both species were strongly negatively linked to some or all components of N deposition (Table 2), though based on multimodel inference the dry deposition of NO_x was emphasized as the key independent variable (CAWs of 0.97 and 0.94 for *S. vesuvianun* and *P. saxatilis*, respectively; Figures 2e,h and 6c). δ^{15}N values declined by 6.3 and 4.6‰ across the N deposition gradient in *S. vesuvianun* and *P. saxatilis*, respectively. Note that the highest mean δ^{15}N value in *S. vesuvianum* was −4.32‰ (Site 8), while in excised cephalodia (from Site 5) it was 0.76 ± 1.8 (n=10, ±1 SE).

3.3 | Archive material

The eight herbaria consulted contain >500 specimens of *S. vesuvianum* originating from Great Britain. Of these, many were collected
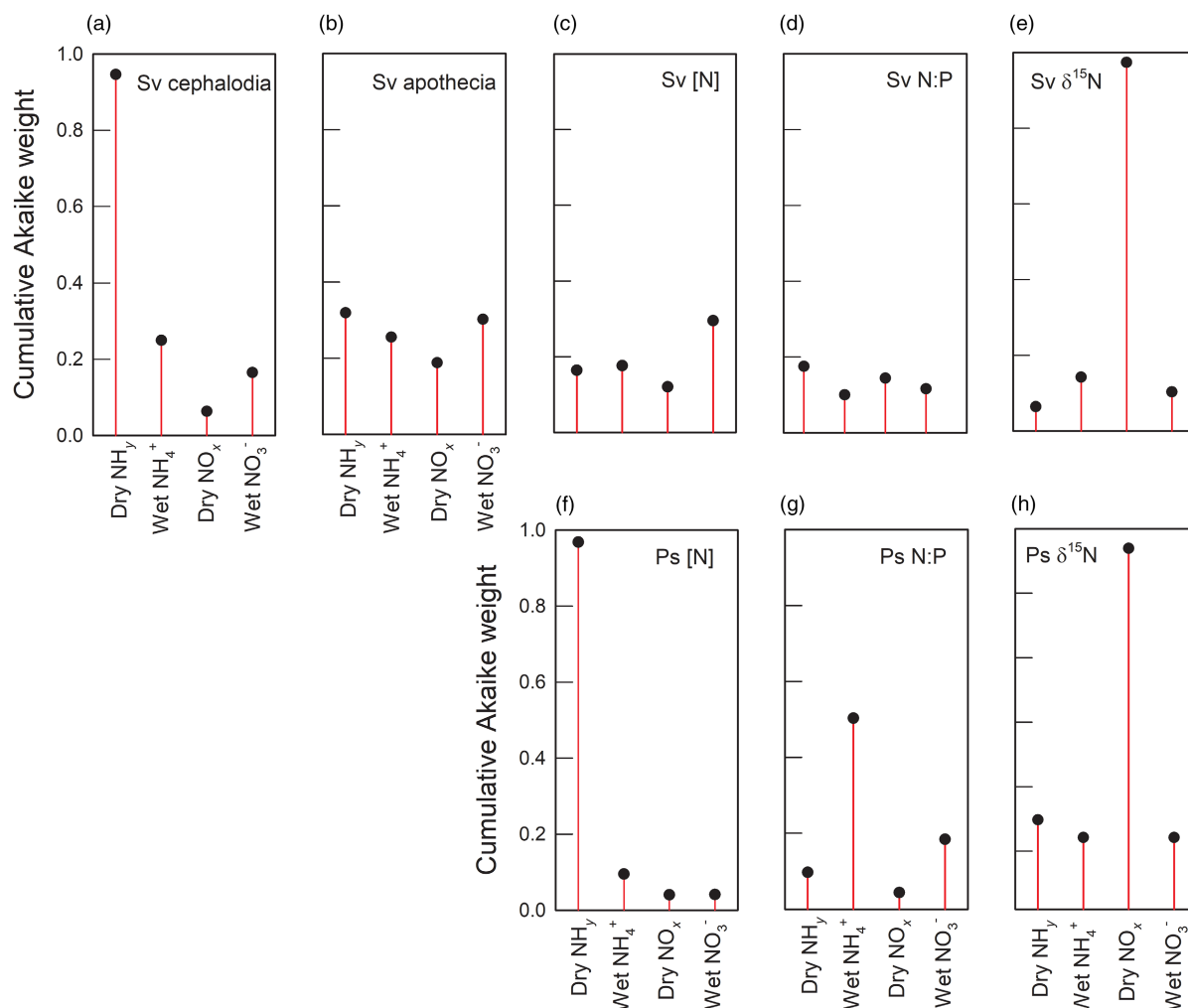
FIGURE 2 The explanatory strength of different components of N deposition scored as their cumulative Akaike weights (multimodel inference) for regression models describing the response of lichen morphology and chemistry. Higher scores indicate N deposition components that tend to feature in the strongest regression models. Sv = *Stereocaulon vesuvianum* (a)–(e), Ps = *Parmelia saxatilis* (f) –(h). (a) and (b), abundance of cephalodia and apothecia, respectively; (c) and (f), thallus [N]; (d) and (g), thallus [N] : [P] mass ratio; (e) and (h), thallus $\delta^{15}N$.

in the 19th century, the majority of which are cephalodiate, often abundantly so. A specimen was noted as having cephalodia only when structures with the verrucose morphology and dark colour that are characteristic of cephalodia in this species could be unambiguously identified. Thus, the absence of cephalodia was uncertain in several old specimens in a poor state of preservation and these records were ignored.

Among the herbaria were several specimens of *S. vesuvianum* collected from, or in the vicinity of, present day N-polluted sites examined in this study. Five of seven specimens collected from Cadair Idris (Site 8) between 1866–1892, two of three specimens collected from Snowdon (7 km NE of Site 6) between 1854–1870, one of two specimens from Helvellyn (4.5 km NE of Site 12) collected in 1883, and one of two specimens collected from Black Craig, Galloway

(18.5 km SSE of Site 11) in 1882 were cephalodiate; cephalodiate specimens are today rare or absent at these sites. Several additional sites at which cephalodiate specimens were collected in the 19th century were revisited in the present study largely confirming that current populations of *S. vesuvianum* therein lack cephalodia (Figure 7a). For example, six of nine specimens from southwest England collected between 1877–1918 were cephalodiate; these included two from locations on Dartmoor where present day populations lack cephalodia (Figure 7a, Table S3). In broad terms it became apparent that most specimens from Great Britain collected prior to 1900 were cephalodiate (Figure 7b) while most specimens from England, Wales and southern Scotland collected after 1940 lacked cephalodia (Figure 7a, Table S4). Among the overseas accessions examined, abundantly cephalodiate specimens of *S. vesuvianum* were
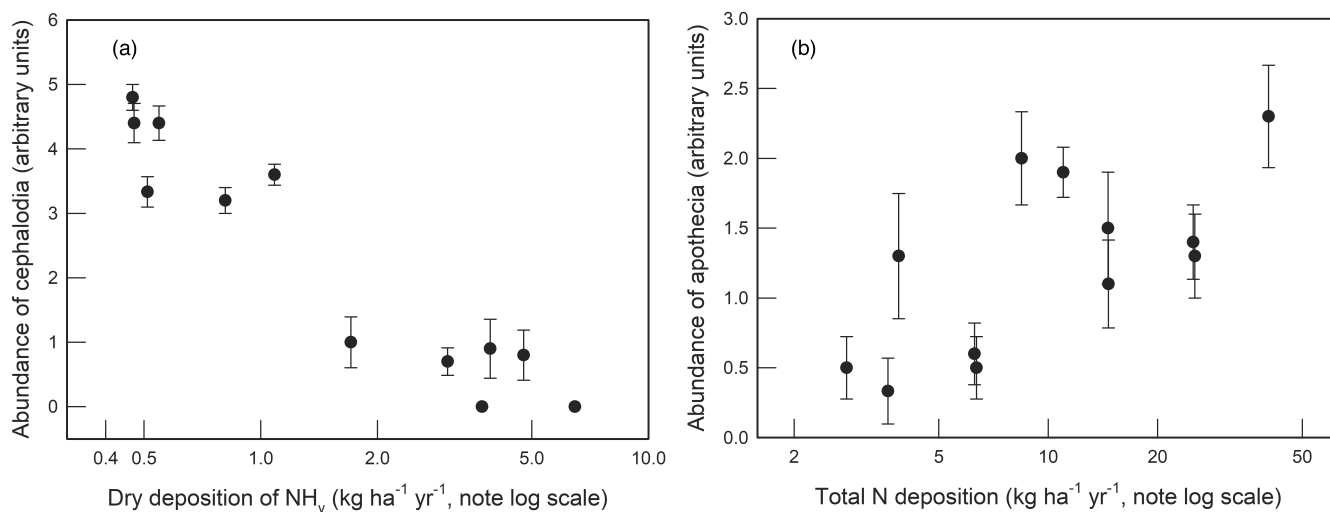
FIGURE 3 Relationships in *Stereocaulon vesuvianum* between N deposition and abundance of (a) cephalodia ($r^2 = 0.90$, $p = <0.001$) and (b) apothecia ($r^2 = 0.44$, $p = 0.019$). Mean values ($n = 10$) are plotted ±1 SE. The order of sites along the *x* axis in (a) is, from left to right, 3, 1, 2, 5, 4, 7, 9, 11, 12, 8, 6 & 10.
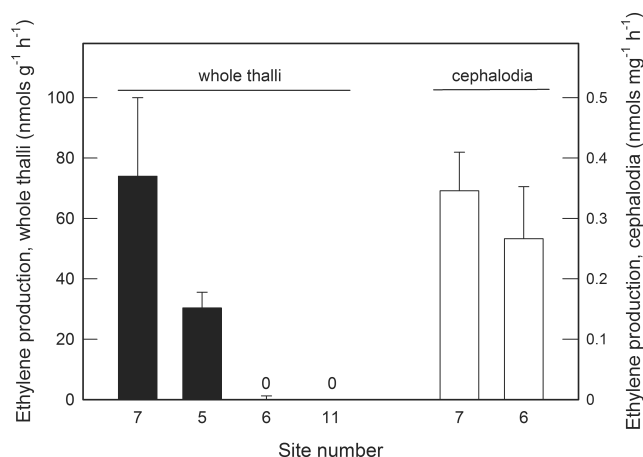

FIGURE 4 Rates of nitrogenase activity (acetylene reduction) in *Stereocaulon vesuvianum*. Assays performed on whole thalli (black columns) with abundant cephalodia (sites 7 & 5) and without cephalodia plus algal biofilm (sites 6 & 11), and on excised cephalodia (open columns) from Site 7 where cephalodia were abundant and from Site 6 where cephalodia were rare. Mean values ($n = 10$) are plotted ±1 SE.

noted from the French Alps, Pyrenees, Madeira, Canary Islands and southern Italy.

4 | DISCUSSION

4.1 | Changes in morphology and N₂-fixing capacity

In unpolluted regions of Britain the tripartite lichen *Stereocaulon vesuvianum* typically produces cephalodia, usually in abundance, containing *Stigonema* sp. and fixes dinitrogen. However, over much
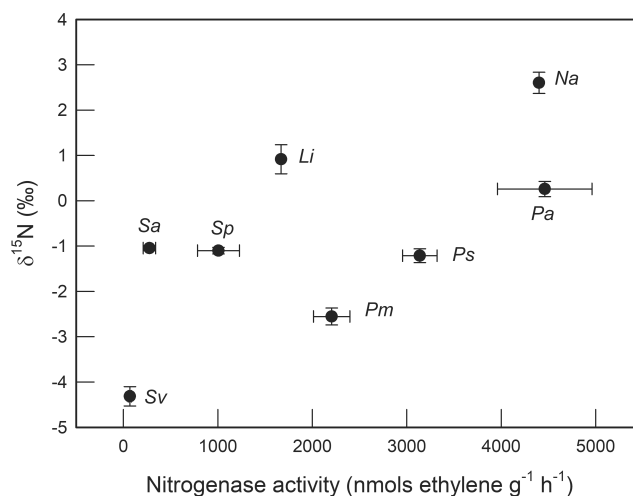

FIGURE 5 Relationship between rate of nitrogenase activity and ^{15}N natural abundance in a range of N₂-fixing lichens. Mean values are plotted ±1 SE (for δ^{15}N, $n = 9$–10); $r^2 = 0.42$, $p = 0.083$). *Li = Lobaria linita*, *Na = Nephroma arcticum*, *Pa = Peltigera aphthosa*, *Pm = P. malacea*, *Ps = P. scabrosa*, *Sa = Stereocaulon alpinum*, *Sp = S. paschale*, *Sv = S. vesuvianum*. δ^{15}N values were measured in the present project. Sources of maximum rates of nitrogenase activity recorded under field conditions were as follows: *Lobaria linita* = Alexander et al. (1974) (Alaska ($n = $ unknown)); *Nephroma arcticum* = Granhall and Selander (1973) (Northern Sweden ($n = 3$)); *Peltigera aphthosa* = Kallio et al. (1976) (Northern Finland ($n = >20$)), *P. malacea* and *P. scabrosa* = P. D. Crittenden and M.-M. Kytöviita (unpublished data; Northern Quebec, Canada ($n = 10$)); *Stereocaulon alpinum* and *S. vesuvianum* = Crittenden (1975) (Southern Iceland ($n = 5$)); *S. paschale* = Crittenden and Kershaw (1979) (Northwest Territories, Canada ($n = 10$)).

of England, Wales and southern Scotland, cephalodia in this species are either rare or absent (Figures 3a and 7a). Cephalodial abundance is strongly negatively correlated with N deposition and is a
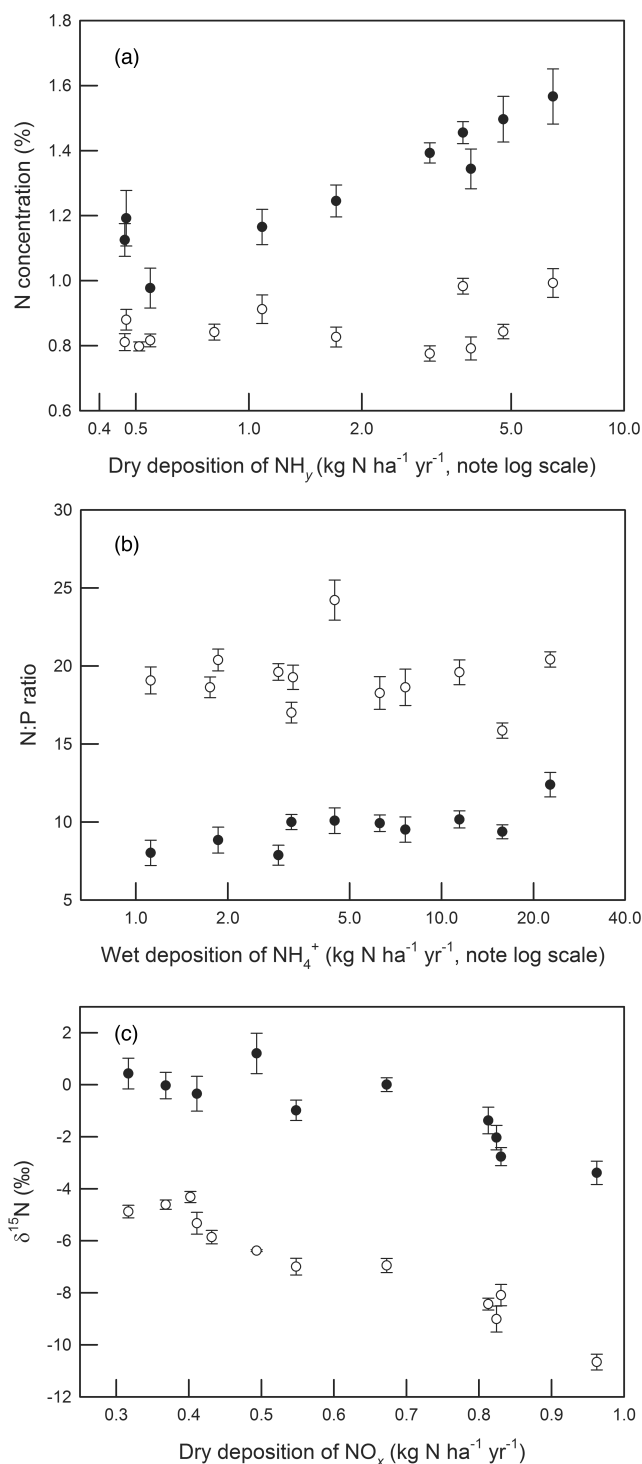
FIGURE 6 Relationships in *Stereocaulon vesuvianum* (○) and *Parmelia saxatilis* (●) between N deposition and (a) thallus N concentration (respectively: $r^2 = 0.14$, $p = 0.23$; $r^2 = 0.85$, $p = <0.001$), (b) thallus N:P mass ratio (respectively: $r^2 = 0.01$, $p = 0.73$; $r^2 = 0.57$, $p = 0.012$) and (c) the natural abundance of ^{15}N (respectively: $r^2 = 0.93$, $p = 0.<001$; $r^2 = 0.71$, $p = 0.002$). Mean values ($n = 10$) are plotted ± 1 SE.

good proxy for the N_2-fixing capacity of *S. vesuvianum* since cyanobacteria are not among the major components of the green algal biofilms that commonly develop on the thallus surface in polluted

regions, and cephalodia that develop infrequently at a moderately polluted site had rates of nitrogenase activity similar to those at a background site. The presence or absence of cephalodia, together with other morphological traits identified in this study as being linked to N deposition, provides an indicator for the degree of N pollution in montane areas of Britain. Thus *S. vesuvianum* in which cephalodia are frequent to abundant, apothecia are infrequent to rare, phyllocladia are predominantly wart-like with small dark centres and algal biofilms are absent or infrequent, occurs in regions subject to annual mean dry deposition of $NH_y \leq 1$ kg ha^{-1} year^{-1}, total reduced $N \leq 6$ kg ha^{-1} year^{-1} and total $N \leq 8$–9 kg ha^{-1} year^{-1}; this is roughly at or below the estimated critical load for total N deposition to subalpine/alpine acid grassland and moss/lichen dominated mountain summits of 5–10 kg ha^{-1} year^{-1} according to Bobbink and Hettelingh (2011).

NH_y, of which NH_3 accounts for roughly 50%, was identified as the principal component of N deposition linked to loss of cephalodium development. There is evidence to suggest that lichens capture NH_3 efficiently. Using flux chamber measurements, Jones et al. (2007) found that hydrated lichens and mosses were stronger sinks for NH_3 than vascular plants and that canopy resistance to deposition was inversely related to $[NH_3]$ values. Furthermore, Sheppard et al. (2011) demonstrated that NH_3 gas is more toxic to lichens and mosses than wet deposited N. While intensive agriculture in lowland areas provides a stronger emission source for NH_3 than upland pastures, Grace and Unsworth (1988) point out that high wind speeds and aerodynamically rough terrain in montane areas lead to greater efficiency of pollution deposition compared to lowland regions. On the other hand, little is known about relationships between lichens and aerosol-bound NH_4^+, the other component of NH_y.

Several factors potentially confounding the relationship between N_2-fixing capacity and N deposition can be dismissed. First, cephalodial abundance is not significantly correlated with H^+ deposition (Tables S1 and S2), which is an inhibitor of N_2-fixation in lichens (Kytöviita & Crittenden, 1994, and references cited therein) and implicated as a factor responsible for the loss of pollution sensitive N_2-fixing epiphytic lichens in many parts of Europe (Farmer et al., 1992; Gauslaa, 1995; Gauslaa & Holien, 1998). Cephalodial abundance is, however, positively correlated with latitude but examination of herbarium specimens suggests that this is unlikely to reflect a causal climatic variable: many specimens collected in the 19th century from regions of Britain that are polluted today were cephalodiate, often abundantly so (Figure 7b), demonstrating that loss of N_2-fixing capacity has occurred relatively recently. The link with latitude probably reflects the strong correlation between latitude and N deposition (Table 2). It is also unlikely that loss of N_2-fixing capacity is a result of recent climate warming since collection site annual mean temperature was only weakly, but *positively*, correlated with cephalodial abundance ($r = 0.56$, $p = 0.06$). The range of annual mean temperatures among the collection sites for the period 2010–2019 was 6.4–9.3°C reflecting the range of site altitudes (for the mid-range altitude for collection spots at each site and site
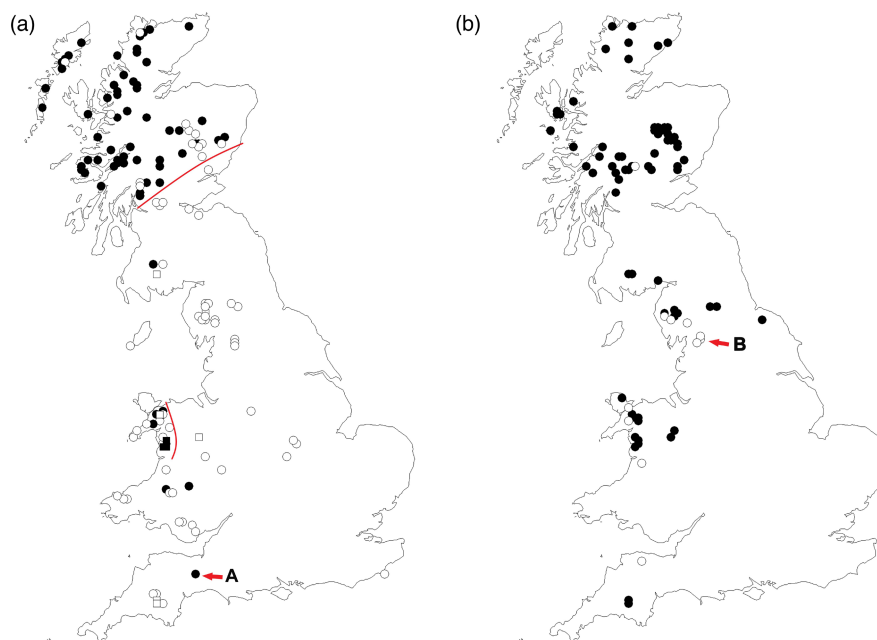
FIGURE 7 Distributions of cephalodiate (solid symbols) and acephalodiate (open symbols) specimens of *Stereocaulon vesuvianum* collected (a) post 1940 (circles = herbarium specimens and specimens collected during the present project, squares = specimens collected during the present project at locations for which pre-1900 cephlalodiate specimens exist in herbaria), and (b) pre 1910 (circles = herbarium specimens). See Table S3. Arrow at 'A' indicates a cephalodiate specimen collected in 1940; arrow at B indicates a cluster of six acephalodiate specimens collected between 1903–06 in northernmost Lancashire. Red lines in (a) identify the principal boundaries between cephalodiate and acephalodiate populations. The year 1910 was selected to circumscribe the records in (b), as opposed to 1900, in order to include the specimens from 'B'.

annual mean temperature, $r = -0.82$, $p = 0.0012$). Moreover, site temperature was not correlated to latitude (Table S1). Note that N$_2$-fixing populations of *S. vesuvianum* occur in putatively warmer montane habitats at more southerly latitudes than those of Britain including a location in southern Italy with an annual mean temperature roughly twice those at the British sites (Note S1). However, this is not necessarily evidence of resilience to modest climate warming since local lichen populations are likely to be adapted to local/regional environmental conditions (cf. Murtagh et al., 2002) and could still be affected detrimentally at temperatures that exceed their capacity to acclimate.

Rates of nitrogenase activity reported here for *S. vesuvianum* assayed under laboratory conditions (30–74 nmol C_2H_4 g^{-1} h^{-1}, assay temperature = 19.5–24°C) compare favourably with those measured in the field in southern Iceland (Crittenden, 1975; 19–69 nmol g^{-1} h^{-1}, assay temperature = 16–17.5°C). P.D. Crittenden and M. Piercey-Normore (unpublished data) measured nitrogenase activity in excised cephalodia from >100 specimens of *S. vesuvianum* collected in northern Manitoba and observed rates ranging from 0–1.73 nmol C_2H_4 mg^{-1} h^{-1}; the mean rate of 0.27 ± 0.04 nmol mg^{-1} h^{-1} ($n = 107$) agrees with rates of 0.26–0.35 nmol C_2H_4 mg^{-1} h^{-1} recorded in the present work (assay conditions: 20°C and 100 μmol m^{-2} s^{-1} PPFR for Manitoba material; 22–23°C and 170–200 μmol m^{-2} s^{-1} PPFR in the present work). Nonetheless, rates of nitrogenase activity in *S. vesuvianum* are relatively low compared to other macrolichens (Figure 5). For example, the mat-forming species *S. paschale* can achieve

rates >1000 nmol C_2H_4 g^{-1} h^{-1} (Crittenden & Kershaw, 1979) while rates >5000 nmol C_2H_4 g^{-1} h^{-1} have occasionally been reported in *Peltigera* spp (Granhall & Selander, 1973; Kershaw, 1974; MacFarlane & Kershaw, 1977; Stutz & Bliss, 1975). Low nitrogenase activity in *S. vesuvianum* (19–74 nmol C_2H_4 g^{-1} h^{-1}) probably reflects a modest N demand in this species linked to low growth rates and thallus [N] values (0.7%–0.8% of thallus dry mass; Crittenden, 1975; present work). There are currently no data available for growth rate in *S. vesuvianum*. However, Kurina and Vitousek (1999) have measured growth rate in *S. vulcani* which has a mode of growth similar to that of *S.vesuvianum* i.e. forming compact tufts firmly attached to rock. Annual extension growth of *S. vulcani* pseudopodetia on lava flows in Hawaii was 2.4–3.8 mm ($n = 200$; =11%–39% increase in thallus length) while nitrogenase activity and thallus [N] values were 6.3–63.1 nmol C_2H_4 g^{-1} h^{-1} and 0.5%, respectively. The higher rates of nitrogenase activity in *S. paschale* are associated with thallus extension of 6.9–7.3 mm ($n = 20$, =14%–15% increase in thallus length) during 15 weeks of the subarctic summer and a thallus [N] value of 1.0%–1.1% (Kytöviita, 1993; Kytöviita & Crittenden, 2007).

The modest rate of N$_2$-fixation in *S. vesuvianum* perhaps underlies its apparent sensitivity to N enrichment i.e. a low rate of N input to the thallus N economy from cephalodial N$_2$-fixation is more likely supplanted by an elevated N supply from atmospheric deposition. The effect of N enrichment on lichens with higher rates of N$_2$-fixation might be more difficult to detect. Indeed, Pentecost (2021) found no difference between a low and a high N deposition site in Britain in

cephalodial abundance in the epiphytic foliose lichen *Lobaria pulmonaria*, a species in which rates of nitrogenase activity are up to two orders of magnitude greater than those in *S. vesuvianum* (Horstmann et al., 1982), while Palmqvist et al. (2017) reported that spraying the terricolous lichen *Peltigera aphthosa* (cf. Figure 5) with a 2.5 mM solution of NH_4NO_3 (a concentration *c.* 50 times greater than the highest mean N concentrations in Swedish precipitation) for 1 min. daily throughout the snow-free growing season reduced cephalodial mass per unit thallus mass by *c.* 30% but did not prevent the formation of new cephalodia. It is noteworthy that the rate of nitrogenase activity in boreal bryophyte-cyanobacterium associations, which is similarly inhibited by N enrichment (see Section 1 above), is comparable to that in *S. vesuvianum* on a per unit mass of moss thallus basis, i.e. <50 (–70) nmol C_2H_4 g^{-1} h^{-1} (Gundale et al., 2009, 2011, 2012; Kox et al., 2016; Leppänen et al., 2013; Rousk et al., 2014; Stuiver et al., 2015).

Failure of cephalodia to develop might result from a loss of response in the N-replete lichen-forming fungus to contact with compatible *Stigonema* cells. It is also possible that (i) algal biofilms over the surfaces of thalli inhibit such interactions, although not all pseudopodetia within thalli in N-polluted regions are contaminated with a biofilm, and (ii) compatible species/strains of *Stigonema* occur less frequently in eutrophicated regions.

4.2 | Variation in thallus chemistry

The loss of N_2-fixing capacity in *S. vesuvianum* might be reflected in changes in thallus ^{15}N natural abundance. The maximum $\delta^{15}N$ value in *S. vesuvianum* of –4.3‰ (Site 2), might indicate that N_2-fixation (providing N with a $\delta^{15}N$ value of *c.* –1‰ (Zhang et al., 2014)) supplies only a part of the lichen's growth-led demand for N and that some ^{15}N-depleted combined N is taken up from the atmosphere and/or other sources; the high $\delta^{15}N$ value of excised cephalodia (0.76‰) is in line with this suggestion. The modest rates of nitrogenase activity in *S. vesuvianum* are also generally consistent with a negative $\delta^{15}N$ value (Figure 5). While loss of N_2-fixing capacity might contribute to the rapid decline in $\delta^{15}N$ values between sites 2–9 along the N deposition gradient (to *c.* –7‰, Figure 6c), cephalodia are rare or absent at the more N-polluted sites and, hence, subsequent decline in $\delta^{15}N$ to *c.* –10.7‰ at Site 8 is probably not a result of further loss of diazotrophic capacity; indeed, relationships in the non-N_2-fixing *P. saxatilis* between thallus $\delta^{15}N$ and N deposition are also negative (Table 2, Figure 6c). The most obvious explanation for these trends prima facie is that pollution-N is ^{15}N-depleted and that both species take up greater quantities of ^{15}N-depleted N as N deposition increases; i.e. in *S. vesuvianum* the effects of cessation of N_2-fixation and uptake of ^{15}N depleted N from the atmosphere are additive in Figure 6c. Dry deposition of NO_x and NH_y were the strongest correlates for thallus $\delta^{15}N$ values in *S. vesuvianum* pointing to gaseous and/or particulate dry deposition being key factors involved in modifying the morphology and chemistry of this species. However, multi-model inference

identified dry deposition of NO_x as the principal influence on thallus $\delta^{15}N$ values in both *S. vesuvianum* and *P. saxatilis*.

In order to more fully interpret the observed geographical variation in ^{15}N natural abundance in both lichen species, data for $\delta^{15}N$ values for atmospheric N deposition are required. Unfortunately, there is a dearth of such isotopic measurements from Britain and, indeed, rather few from Europe more widely (Table S4). However, there is a relative abundance of information from other populous regions, particularly North America and East Asia. Data from these regions suggest that, while there is wide variation among measurements, there is a general trend towards wet-deposited NH_4^+ and NO_3^-, and dry deposition NH_3 and NO_2 being ^{15}N depleted while aerosol-associated N is ^{15}N enriched. There is also a tendency in wet deposition for NH_4^+ to be more ^{15}N depleted than NO_3^-, and in dry deposition for gaseous NH_3 to be more ^{15}N depleted than NO_2 (NH_3 and NO_2 of agricultural origin being particularly ^{15}N-depleted; Bhattarai et al., 2021; Elliott et al., 2019; Heaton, 1986; Huang et al., 2019; Kendall et al., 2007; Ti et al., 2018; references summarized in Table S4). Negative $\delta^{15}N$ values for both *S. vesuvianum* and *P. saxatilis* in N polluted regions are thus generally in line with available data for N deposition. However, this summary does not account for the apparent strong link between lichen $\delta^{15}N$ values and NO_x deposition rate. Indeed, at first sight this link appears anomalous given that at polluted sites modelled deposition rates for NH_y were up to 8 times greater than those of NO_x (Table 1).

Capture of NH_3 and NO_2 for the purposes of concentration and ^{15}N natural abundance measurements is most frequently achieved using passive samplers (Table S4). Recently, Pan et al. (2020) have shown that such samplers underestimate NH_3 $\delta^{15}N$ values by 15.4‰ owing to the lower rate of diffusion of the heavier N-related isotopologue to absorptive surfaces (a value that the authors point out approaches the theoretical diffusive isotope fractionation of 17.7‰). The error associated with passive sampling of NO_2 was not measured but the theoretical value (4.1‰) is smaller owing to the lower relative mass difference between N-related isotopologues of NO_2 compared to NH_3. Further, available data indicate that aerosol-associated NH_4^+, a putative $\delta^{15}N$ positive component, accounts for roughly 50% of [NH_y] measurements in our study areas. These influences on the $\delta^{15}N$ signatures of NH_y versus NO_x raise the possibility that while [NH_y] values in polluted regions are higher than those for [NO_x], and could be important in determining thallus N concentrations, NO_x, comprising roughly 80% NO_2, might be more ^{15}N depleted than NH_y and an important determinant of thallus $\delta^{15}N$ values (cf. Moore, 1977). ^{15}N:^{14}N ratios in NO_2 are dependent on emission sources but are also modified by isotopic fractionations during chemical reactions in the atmosphere (Li et al., 2020). Of these, photochemical cycling is likely to dominate at low [NO_2] values such as might occur in remoter upland regions, resulting in further ^{15}N depletion of up to 10‰ (Li et al., 2020; Walters et al., 2016). More critical assessment of this question will require isotopic data for N deposition in UK airsheds. Notwithstanding these speculations, Tozer et al. (2005) suggested that the strongly negative $\delta^{15}N$ signatures in epiphytic lichens in New Zealand rain

forests might result from discrimination against ^{15}N during diffusive uptake of NH_3.

While the relative importance of NH_3 and NO_2 in influencing lichen thallus ^{15}N content remains uncertain, the emphasis on dry deposited N as a putative determinant of both lichen chemistry and N_2-fixing capacity is nonetheless noteworthy. In areas where cephalodiate material of *S. vesuvianum* was generally rare or absent, specimens with cephalodia could occasionally be encountered in sheltered habitats such as ravines. Such a distribution is consistent with a role of dry deposition of N in the inhibition of cephalodium formation. Gilbert (1970) earlier observed that shelter could amelio-rate the toxic effects of SO_2 pollution on lichens.

Correlations between NH_y deposition and lichen $\delta^{15}N$ values reported here (Table 2) have been identified previously by other workers. Boltersdorf and Werner (2013, 2014) and Boltersdorf et al. (2014) found that $\delta^{15}N$ values in epiphytic lichens sampled in northwest Germany were in the range −15 to −1‰ and negatively related to proxies for NH_3 deposition and the ratio NH_4^+-N:NO_3^--N in wet deposition (but see Boltersdorf et al., 2014). Similar results were obtained by Solga et al. (2005) for pleurocarpous mosses sampled in the same region and by Bragazza et al. (2005) for *Sphagnum* sampled more widely across Europe. However, these studies did not consider relationships between $\delta^{15}N$ signatures and NO_x deposition. Interestingly, these negative relationships between N deposition and $\delta^{15}N$ values observed in lichens and bryophytes are the inverse of those observed in the foliage of forest trees (Emmett et al., 1998; Pardo et al., 2007; i.e. in trees, ^{15}N natural abundance is positively correlated with N deposition) likely reflecting microbial N transfor-mations in soil causing losses of ^{15}N-depleted N, overprinting and inverting the ^{15}N signatures of deposited N (Pardo et al., 2007).

The strong relationship in *P. saxatilis* between thallus [N] and the rate of N deposition (Figure 6a) is consistent with numerous reports that lichen thallus [N] is a coherent indicator of N load (Boltersdorf et al., 2014; Bruteig, 1993; Geiser & Neitlich, 2007; Hogan et al., 2010; Hyvärinen & Crittenden, 1998). The increase in thallus [N]:[P] mass ratio with increasing N deposition (Figure 6b) suggests that P availability probably does not increase along the N pollution gradient. It is perhaps intriguing, therefore, that thallus [N] in *S. ve-suvianum* was not correlated with N deposition (Table 2, Figure 6a). Two factors might underlie this result. First, over the first half of the N deposition gradient, increased uptake of pollution-derived N from atmospheric sources will likely replace, rather than add to, N income from N_2-fixation; a switch from N_2-fixation to increased uptake of combined N without marked effects on growth is a well-known re-sponse to fertilization in legumes (e.g., Bethlenfalvay et al., 1978). Second, there is now compelling evidence that N_2-fixing lichens, in-cluding species of *Stereocaulon*, are P-limited (Benner et al., 2007; Benner & Vitousek, 2007, 2012; Crittenden et al., 1994; Kurina & Vitousek, 1999; McCune & Caldwell, 2009; Weiss et al., 2005). For example, McCune and Caldwell (2009) observed that dipping thalli of *Lobaria pulmonaria* in a 19 mM PO_4^{3-} solution more than doubled its growth rate during the following 12 months. Furthermore, Houlton et al. (2008) have argued that a primary evolutionary advantage of

N_2-fixation in symbiotic systems is an increased capacity for P cap-ture. Hence, in N_2-fixing lichens the maintenance of [N]:[P] stoichi-ometry might govern rates of both nitrogenase activity and uptake of combined N; i.e. luxury consumption of N, as evidenced in *P. saxatilis*, might be disadvantageous in a N_2-fixing system. Increase in growth rate with resulting growth dilution of thallus [N] could also account for the apparent lack of response of thallus [N] to N enrichment but this explanation is inconsistent with a broadly constant [N]:[P] ratio and runs counter to *S. vesuvianum* being P-limited. Indeed, rates of N_2-fixation more generally are thought to be linked to the ratio of available N to available P (Reed et al., 2011). Interestingly, Weiss et al. (2005) found that fertilization with P roughly doubled rates of nitrogenase activity in *Peltigera* spp in pristine arctic tundra. While thallus N:P mass ratio in *S. vesuvianum* is not correlated with cephal-odium abundance (and hence N_2-fixation), the N:P ratio in *P. saxatilis* is, i.e. in environments where the N:P value in P. saxatilis is elevated (> *c*. 10 compared to *c*. 8 at background sites), *S. vesuvianum* largely lacks cephalodia. Again this points to the N:P ratio in *P. saxatilis* re-flecting the availability of N and P in the different habitats sampled while in *S. vesuvianum* it is constrained as argued above. Note that differences between the two species in strategies for N capture in-validates the use of *P. saxatilis* as a non-N_2-fixing reference species in estimating N_2-fixation based on thallus $\delta^{15}N$ values (cf. Dawson et al., 2002).

4.3 | Historical perspective provided by archive material

Examination of archive herbarium specimens revealed that the gra-dient in cephalodium abundance identified in the present survey (Figure 3a) probably did not exist in the 19th century and that loss of cephalodia occurred principally during the first half of the twentieth century. These conclusions align well with the results of retrospec-tive simulations of N deposition in Britain back to 1800 undertaken in Tipping et al. (2017) and Bell et al. (2021). The modelled data suggest that in 1800 a total N deposition of 8 kg ha^{-1} year^{-1}, an ap-proximate critical load for cephalodium formation in *S. vesuvianum* (see Section 4 above), was not exceeded at any of the 12 sampling sites in the present work, whereas in 1900 it was exceeded at two sites (9 & 12) and in 1950 at five sites, compared to seven sites in 2014–16. The distribution of cephalodiate and acephalodiate speci-mens collected post 1940 (Figure 7a) accords with that depicted by cephalodial abundance along the N deposition gradient studied here (Figure 3a). The timing of loss of N_2-fixing capacity no doubt varied among regions of the country. For example, six specimens collected from north Lancashire between 1903–06 were all acephalodiate ('B' in Figure 7b) whereas a specimen collected from south Somerset in 1940 remained cephalodiate ('A' in Figure 7a). During the early part of the 20th century, other toxic pollutants such as SO_2 and acidity impaired growth and survival of lichens in many regions of Britain and it cannot be certain that N enrichment was the primary factor that originally caused the failure of cephalodium development in the

herbarium specimens. Nonetheless, some early acephalodiate herbarium specimens (e.g., from the cluster at 'B', Figure 7b) had abundant apothecia and expanded plate-like phyllocladia, characteristics identified in the present work as indicators of N enrichment. The effect of SO_2 toxicity, on the other hand, is generally to supress sexual reproduction in lichen fungi (Gilbert, 1970).

4.4 | Concluding remarks

This research demonstrates inhibition of N_2-fixation in regions subject to many decades of N enrichment. It is unique in setting this finding against an historical timeline. It also highlights the importance of herbaria as archives of environmental change. In particular, the availability of large numbers of specimens of what is a relatively common species proved invaluable in mapping changes in lichen functionality over both time and space.

The question arises as to whether the contrasting populations of the lichen-forming fungus *S. vesuvanum*, i.e. with and without cephalodia, are genetically distinct. Unfortunately we currently have no data that definitively clarify this point. Cephalodiate specimens of *S. vesuvianum* from Site 5 (low N deposition) and acephalodiate specimens from Site 12 (high N deposition) were included in a phylogenetic analysis of this species conducted by B. McCune (personal communication, December 2017) and were found to have identical ITS sequences in an alignment of 625 bases. However, use of the ITS region alone might not be sufficient to detect small changes elsewhere in the genome. It is probable that loss of cephalodium production probably occurred over too short a period to be explained by population expansions among one or more non-N_2-fixing genotypes. We propose that a more likely explanation for acephalodiate specimens in regions experiencing high N enrichment is that this does indeed impair cephalodium formation consistent with classic models of diazotrophy.

Nitrogen emissions, concentrations in air and precipitation, and deposition rates have all declined during recent decades in Britain (Curtis & Simpson, 2014; Fowler et al., 2005; Monteith et al., 2016; RoTAP, 2012; Tang et al., 2018), continental Europe (Fowler et al., 2007; Oenema et al., 2011) and in North America (Benish et al., 2022). The rates of decline have varied greatly among locations, N species and time intervals examined with the larger decreases tending to occur in areas with higher concentrations and deposition rates. Analyses of N deposition data for Britain from 1986 onwards show that deposition loads of both oxidized and reduced N consistently declined in southern and eastern England by up to 20%, or more, while in western and northern upland regions, which constitute the principal range for *S. vesuvianum* (Figure 1a), deposition trends were more variable and less frequently statistically significant. However, at the interfaces between cephalodial and non-cephalodial populations of *S. vesuvianum* (Figure 7a) even modest declines in N deposition rate could reduce values to below the putative critical load for cephalodial development (*c.* 8–10 kg total N ha^{-1} year^{-1}, see Section 4 above) and result in recovery of N_2-fixing

capacity. The lag time for recovery might be comparatively short since *S. vesuvianum* does not accumulate N by luxury consumption and, therefore, growth dilution of thallus N capital would not be a prerequisite for cephalodium development. If the growth rate of *S. vesuvianum* is close to that of the morphologically correspondent *S. vulcani* (2–4 mm extension per year, see above), then renewed development of cephalodia and recovery of N_2-fixing capacity under these circumstances might perhaps be expected within *c.* 5 years. It is instructive to consider the potentially contrasting circumstances in bulk soil, where rates of N_2-fixation by free-living bacteria are also low (Dawson, 1983; Reed et al., 2011; Wang, Li, et al., 2019) and susceptible to inhibition by N pollution (cf. Ochoa-Hueso et al., 2013). Soils with a long history of N enrichment contain elevated N capital (Perakis et al., 2017; Phoenix et al., 2004; Van Meter et al., 2016); this legacy N could result in substantial lags in recovery of N_2-fixation under conditions of declining N deposition (cf. Gilliam et al., 2019). An extension of the current survey of a montane N_2-fixing lichen at sites subject to contrasting N deposition rates to one of N_2-fixation in bulk soils in semi-natural ecosystems might help to clarify the wider impact of N pollution on N_2-fixation.

AUTHOR CONTRIBUTIONS
Peter D. Crittenden designed the research, obtained the funding, undertook the field, herbarium and laboratory work (except total N and $\delta^{15}N$ measurements), interpreted the results and wrote the paper. Barry Thornton and Wolfgang Wanek made the total N and $\delta^{15}N$ measurements. Rognvald I. Smith provided N deposition data. Christopher J. Ellis downloaded temperature data, provided statistical advice and performed the multiple regression. All authors commented on an earlier version of this manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Raw data used to plot Figures 3–6 have been placed in The University of Nottingham Research Data Repository at https://doi.org/10.17639/nott.7255 (Crittenden et al., 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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