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Standard Paper

On nitrogen fixation in lichens with unicellular cyanobionts

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Abstract

Lichens containing unicellular cyanobionts have received scant attention regarding their nitrogen- (N_2-) fixing capacities. *Thyrea girardii* (*Lichinomycetes*), containing the putative cyanobiont *Chroococcidiopsis*, is the only species in this group so far reported to fix N_2. Nitrogenase activity in *T. girardii* was higher in the light than in the dark, counter to overwhelming evidence that free-living unicells fix N_2 in the dark only, providing temporal separation from oxygenic photosynthesis in the light. The original focus of the current work was to better understand the strategies by which N_2 fixation and photosynthesis might operate concurrently in species of *Lichinella* (*Lichinellaceae*) and *Thyrea* (*Porocyphaceae*) but this aim was frustrated by the infrequency with which nitrogenase activity was detected in the populations sampled. This problem itself became a principal topic of this report. *Lichinella nigritella*, *Thyrea confusa* and *T. girardii* were collected in Catalonia, Spain and subjected to measurements of nitrogenase activity (acetylene reduction) and net CO_2 exchange. Of 91 specimens of the three species combined that were tested, 49 had zero nitrogenase activity and only 23 had activity ≥ 5 nmol C_2H_4 g^{-1} h^{-1} with no obvious trends among species. Nitrogenase activity was consistently greater in the light than in the dark during diel dark-light-dark transitions. Maximum rates of nitrogenase activity recorded for *L. nigritella*, *T. confusa* and *T. girardii* were 133.4, 28.7 and 47.0 nmol C_2H_4 g^{-1} h^{-1}, respectively. All specimens tested were photosynthetically competent, although rates of net CO_2 exchange were comparatively low (maximum rates in *L. nigritella* and *T. girardii* were 37 and 16%, respectively, of maximum rates in *Peltigera membranacea*), and there was no evidence that photosynthetic rates were lower in thalli with appreciable nitrogenase activity. ^{15}N natural abundance values for *L. nigritella* and *T. girardii* ($\delta^{15}N = -3.41$ and -0.84 ‰, respectively) were in agreement with published values for lichens with cyanobacterial photobionts. The results are discussed in relation to possible explanations for the low frequency of nitrogenase activity in these species, its occurrence in the light, and potential confounding factors (the presence of epiphytic heterocytous cyanobacteria and N pollution). It is suggested that the capacity of unicellular cyanobacteria to rapidly switch between non-diazotrophic and diazotrophic states may be advantageous in lichens with unicellular cyanobionts in habitats where N supply is only occasionally limiting.

Keywords: *Lichinales*; *Lichinella*; ^{15}N natural abundance; nitrogenase activity; photosynthesis; physiology; *Thyrea*

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Introduction

All nitrogen (N_2-) fixing bacteria, and symbiotic associations with which they might be involved, must solve the 'oxygen problem' (*sensu* Postgate 1982), viz. that nitrogenase, the enzyme complex responsible for N_2 fixation, is irreversibly inactivated by oxygen (Gallon 1992). This problem is acute in cyanobacteria since they evolve oxygen during photosynthesis. Many filamentous cyanobacteria solve the oxygen problem by the spatial separation of the two processes with N_2 fixation taking place in heterocytes (= heterocysts), specialized cells which lack photosystem II and thus do not assimilate carbon or evolve oxygen, while oxygenic photosynthesis takes place in neighbouring vegetative cells. This enables heterocytous cyanobacteria to assimilate N_2 in the light concurrent with photosynthesis and there is a bi-lateral exchange of carbon (C) and N between heterocytes and adjacent vegetative cells (Herrero *et al.* 2016; Nieves-Morión *et al.* 2021). Most N_2-fixing lichens, and all plant symbioses involving cyanobacteria, contain heterocytous cyanobionts (Adams 2000). There is, however, a range of little-studied lichens, mostly in the *Lichinales* (*Lichinomycetes* (Prieto *et al.* 2024)), that contain unicellular cyanobacteria; these include species in the genera *Anema*, *Lichinella*, *Peccania*, *Peltula*, *Pyrenopsis* and *Thyrea*, several of which are important as members of soil crust communities in arid lands (Belnap & Lange 2003).

Studies on N_2 fixation in free-living unicellular cyanobacteria have been confined largely to species of the terrestrial/freshwater genus *Gloeothece* (= *Gloeocapsa*) (Mareš *et al.* 2019), and to species of *Crocosphaera* (= *Cyanothece* = *Synechococcus*) that are important components of marine nanoplankton communities (Cleveland *et al.* 2024; Masuda *et al.* 2024). When laboratory cultures of these species are grown under light/dark cycles, nitrogenase activity occurs predominantly (but not exclusively, see Discussion) during dark periods thus providing temporal separation of N_2 fixation from oxygenic photosynthesis in the light (Mullineaux *et al.* 1981; Mitsui *et al.* 1986; Reddy *et al.* 1993; Sherman *et al.* 1998; Falcón *et al.* 2004; Mohr *et al.* 2010; Masuda *et al.* 2024). These laboratory observations have been substantiated by *in situ* measurements on

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natural populations of *Gloeothece* growing on limestone (Griffiths *et al.* 1987) and on *Crocosphaera* in the North Pacific Ocean (Zehr *et al.* 2001; Wilson *et al.* 2017), which showed that maximum nitrogenase activity and *nifH* transcript abundance occurred during dark periods. These temporal oscillations in nitrogenase activity appear to be orchestrated by a circadian clock since under laboratory conditions they continue when cultures are transferred to continuous light (Mitsui *et al.* 1986; Schneegurt *et al.* 1994; Shi *et al.* 2010; Cohen & Golden 2015; Bandyopadhyay *et al.* 2024). Prior to and at the onset of photosynthesis, nitrogenase is progressively inactivated, and then subsequently re-synthesized and re-activated *de novo* leading up to and during the ensuing dark period (Gallon & Chaplin 1988; Gallon 1992; Du & Gallon 1993; Sherman *et al.* 1998; Reade *et al.* 1999; Mohr *et al.* 2010; Shi *et al.* 2010; Aryal *et al.* 2011) coupled with an inactivation of PSII (Masuda *et al.* 2018) consistent with a circadian periodicity. By contrast, Crittenden *et al.* (2007) reported that the lichen *Thyrea girardii*, containing the putative unicellular cyanobiont *Chroococcidiopsis* (Büdel & Henssen 1983; Moreno & Egea 1992; Fewer *et al.* 2002), fixed N_2 in the light at rates that were higher than in the dark.

The original aim of the present study was to better understand how concurrent N_2 fixation and photosynthesis in species of *Thyrea* and *Lichinella* might be temporally and/or spatially separated in the lichen thallus. Unfortunately, this aim was frustrated by the low frequency with which nitrogenase activity was detected in the populations sampled. This problem itself then became a principal topic of this account.

Materials and Methods

The species, and their collection and pretreatment

Three lichen species in the order *Lichinales* with unicellular cyanobionts, *Lichinella nigritella*, *Thyrea confusa* and *T. girardii*, were collected from the Barcelona region (Catalonia) in Spain during three field excursions in 2017 (Table 1). These are small foliose to foliose-fruticose heteromerous lichens that form dark grey to black rosettes or cushions up to 20–25 mm in diameter, attached to the substratum by an umbilicus and with warts or globose isidia on the upper surface (Moreno & Egea 1992; Schultz 2002, 2007). Specimens were collected in the air-dry state from seepage faces of limestone or basic sandstone cliffs by being cut at the umbilicus with a scalpel. They were returned to the laboratory within two days and placed in a growth room with a 15 °C/18 °C night/day cycle, a photoperiod commensurate with the Barcelona area at the time of collection and at a photosynthetic photon fluence rate (PPFR) at the thallus surface of *c.* 150 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Most experimental work was completed within 14 days of collection as reported in Supplementary Material Table S1 (available online) which also gives data on the duration of dry periods preceding the collections.

Other lichen species were collected for comparative purposes: *Collema* spp. s. lat. and *Romjularia lurida* from the same cliff faces as *Lichinella* and *Thyrea*, and *Peltigera membranacea* from Great Britain (Table 1). *Collema*, *Romjularia* and other specimens for isotopic analysis were stored air dry at −18 °C; *Peltigera*, after collection, was air-dried on a laboratory bench at 18 °C and then placed in a growth room as above with a photoperiod appropriate for the collection site.

Measurement of nitrogenase activity and net CO_2 exchange

Air-dry lichen thalli were rehydrated *c.* 14 h prior to assays by being placed on foam rafts covered with Whatman No. 1 filter paper, which dipped into deionized water, and then placed under growth room conditions. Prior to assays, all surfaces of each hydrated thallus were thoroughly but gently 'scrubbed' in water under a dissecting microscope using an artist's paintbrush with the hairs cut to a length of *c.* 3–4 mm, aided by a water jet from a syringe fitted with a blunt needle, in an attempt to remove adhering epiphytic cyanobacteria and other contaminating debris, mostly mineral particles.

Table 1. Lichen species studied, collection sites and dates, and number of specimens in which zero, trace, low and high rates of nitrogenase activity were recorded (zero = gas chromatograph output < limit of detection; trace = gas chromatograph output > detection limit but nitrogenase activity < 5 nmol C_2H_4 g^{-1} h^{-1}; low = nitrogenase activity > 5 and < 20 nmol C_2H_4 g^{-1} h^{-1}; high = nitrogenase activity > 20 nmol C_2H_4 g^{-1} h^{-1}).

Species	Collection sites* and dates		Number of specimens with nitrogenase activity categories zero/trace/low/high
Lichinella nigritella (Lettau) P.P. Moreno & Egea	Spain 1 & 2	2 & 3 March 2017	1/2/5/0
	Spain 1,2 & 3	28 & 29 September 2017	4/1/1/0
	Spain 2 & 4	27 & 28 November 2017	4/3/2/4
Thyrea confusa Henssen	Spain 2 & 4	27 & 28 November 2017	3/7/4/1
T. girardii (Durieu & Mont.) Bagl. & Carestia	Spain 1 & 2	2 & 3 March 2017	8/2/1/3
	Spain 1,2 & 3	28 & 29 September 2017	8/2/0/0
	Spain 2 & 4	27 & 28 November 2017	21/2/2/0
Collema spp. s. lat.†	Spain 2	15 June 2021	—
Peltigera membranacea (Ach.) Nyl.	GB 5	2 February 2017	0/0/0/8
Romjularia lurida (Ach.) Timdal	Spain 1 & 2	27 & 28 November 2017	—

*Collection sites
1. Limestone cliffs, Olèrdola Nature Park, near Vilafranca del Penedès (41°18′35″N, 1°39′52″E)
2. Limestone cliffs, by River Foix, near Pantà de Foix (41°14′45″N, 1°42′35″E)
3. Calcareous sandstone cliffs, near Torà (41°50′00″N, 1°27′02″E)
4. Limestone cliffs, Montserrat, by path to la Santa Cova (41°35′24″N, 1°50′21″E)
5. Moss covered limestone walls, Musden Wood, near Ilam (53°03′26″N, 1°49′30″W)
†Probably includes specimens of *Enchylium polycarpon* (Hoffm.) Otálora *et al.* (= *Collema polycarpon*) and *Lathagrium cristatum* (L.) Otálora *et al.* (= *Collema cristatum*)

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Nitrogenase activity was quantified by the acetylene reduction method (Stewart *et al.* 1967). Each thallus was placed in a 17 ml capacity glass cuvette (70 ml in the case of *Peltigera*) which was then sealed with a Suba-Seal septum. Acetylene was then introduced to form a 10% concentration by volume and the cuvette placed under a fluorescent light bank at temperature and PPFR values measured inside closed cuvettes of 18–22 °C and 170–200 μmol m^{-2} s^{-1}, respectively. After *c.* 1 h the ethylene content of the headspace gas was determined by gas chromatography following Crittenden *et al.* (2023). 'No lichen' and 'no acetylene' controls were included in each series of assays.

Note that assays used commercially available acetylene gas, the contaminant ethylene content of which was reduced following Witty (1979). The limit of detection for ethylene, calculated after Wilson (1974) as 5 × standard deviation of blank values (= at the 95% confidence level), varied between batches of purified acetylene with a mean value of 2.71 ± 0.46 pmol (± 1 SE, n = 8), roughly equivalent to 1.96 ± 0.31 nmol C_2H_4 g^{-1} h^{-1} for a typical thallus dry mass of 50 mg and a 1 h incubation.

Net CO_2 exchange was measured following the method of Larson & Kershaw (1975; see also Busby & Whitfield (1978)). This involved incubating thalli in closed cuvettes (70 or 100 ml capacity, depending on species) under the laboratory conditions described above. Cuvettes were flushed with cylinder air and 0.5 ml subsamples of cuvette headspace gas removed at the start and end of the incubation period (typically 7–12 min). CO_2 in the gas subsamples was measured by gas chromatography using a Pye Unicam 4500 GC fitted with a nickel catalyst following the method of Crittenden & Kershaw (1978). CO_2 concentrations in cuvettes did not fall below *c.* 200 ppm (*c.* 8 nmol CO_2 ml^{-1}).

In all assays thallus wet mass was measured at both the start and end of each incubation period, and at the end of each run thallus oven dry mass was determined after 24 h at 80 °C. The mean dry mass values (± SE) of thalli used in assays were 59 ± 5 mg for *L. nigritella* (n = 36), 53 ± 4 mg for *T. confusa* (n = 15), 73 ± 4 mg for *T. girardii* (n = 52) and 25 ± 2 mg for *P. membranacea* (n = 23). At all stages of set-up and with the introduction and removal of gases, care was taken to maintain the cuvette headspace gas at atmospheric pressure.

On some occasions, sequences of net CO_2 exchange and/or nitrogenase activity measurements were performed. When these sequences involved dark to light transitions, the first assays were performed in the dark, in which case the selected thalli were removed from the growth room during the late dark period with great care being taken to maintain the material in darkness until the set time to initiate illumination. In these experimental runs, thallus water content was maintained ±constant by adding water to a set target mass at the start of each assay. When both nitrogenase activity and net CO_2 exchange were measured concurrently, these two assays were performed alternately on the same samples. In other sequential measurements, net CO_2 exchange was monitored as thalli progressively dried.

Determination of ^{15}N natural abundance and total N concentration

Air-dried lichen thalli were cleaned of adhering surface debris with a dry artist's paintbrush (modified as above), oven dried at 40 °C and then reduced to powder in a ball mill. The powder was re-dried and subsamples weighed into ultra-clean tin capsules (Elemental Microanalysis Ltd). Total thallus N and its ^{15}N:^{14}N ratio 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 the method are provided in Crittenden *et al.* (2023).

Microscopic examination of thalli

When all nitrogenase and net-CO_2-exchange measurements were completed, selected thalli (six from each class of nitrogenase activity) were subjected to detailed microscopic examination to determine the extent, if any, of contamination by heterocytous cyanobacteria. Hydrated thalli were dissected into separate component lobes and the surfaces of each were examined with a dissecting microscope at ≤ × 80 (Leica S8Apo). Putative cyanobacterial contamination from each lobe was then further examined under high power magnification ≤ × 603 (Leica DM2000). For each lobe within a thallus, hand-cut vertical sections were prepared from which squash preparations were made and examined under high power for the presence of heterocytous cyanobacteria; squash preparations of isidia were likewise examined.

Data analysis

Where appropriate, data were subjected to the Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test, or to correlation analysis, both performed in SigmaPlot v. 11 (Systat Software Inc.), or to *t*-tests. Data were checked for normality and homogeneity of variances; where test assumptions were not met, data were log-transformed. Curve fitting was undertaken using HPcurves (Parsons & Hunt 1981; available at https://www.roderick-hunt.co.uk/science-growth-analysis). This program fits a splined series of curves to the natural logarithms of sequential determinations of variates, 95% confidence limits being calculated for each of the resultant fitted values. In the present work, fitted values and 95% confidence limits were back-transformed from logarithms.

Results

A total of 91 specimens of *Thyrea confusa*, *T. girardii* and *Lichinella nigritella* combined were assayed for nitrogenase activity. Of these, 49 specimens had zero (not detectable) activity, 19 had trace, 15 low and eight high activity (Table 1). Definitions of these classes of enzyme activity are provided in Table 1. There were no obvious trends in activities among species or collection dates. Many specimens were assayed only once while others were subjected to a sequence of measurements, for example during 11–14 h dark-light-dark (night-day-night) diel transitions (Figs 1, 2A & 3A). Nitrogenase activities during these series of measurements were generally accordant (i.e. uniformly zero, trace, low or high); marked changes in activity in the same specimen were seldom observed. The consistent measurements of trace levels of activity (Figs 1B, 2A & 3A) suggest that these were not the result of measurement error. The highest nitrogenase activities recorded for *Thyrea confusa*, *T. girardii* and *Lichinella nigritella* were 28.7, 47.0 and 133.4 nmol C_2H_4 g^{-1} h^{-1}, respectively; these values can be compared with a maximum of 3870 nmol C_2H_4 g^{-1} h^{-1} in *Peltigera membranacea.* Specimens found to have high activity in the first assay were cleaned by brushing for a second time in an attempt to further minimize interference from adhering epiphytic heterocytous cyanobacteria, but this did not result a decline in subsequent activity (e.g. Figs 1, 2B & 3A). Several environmental factors were varied in unsuccessful

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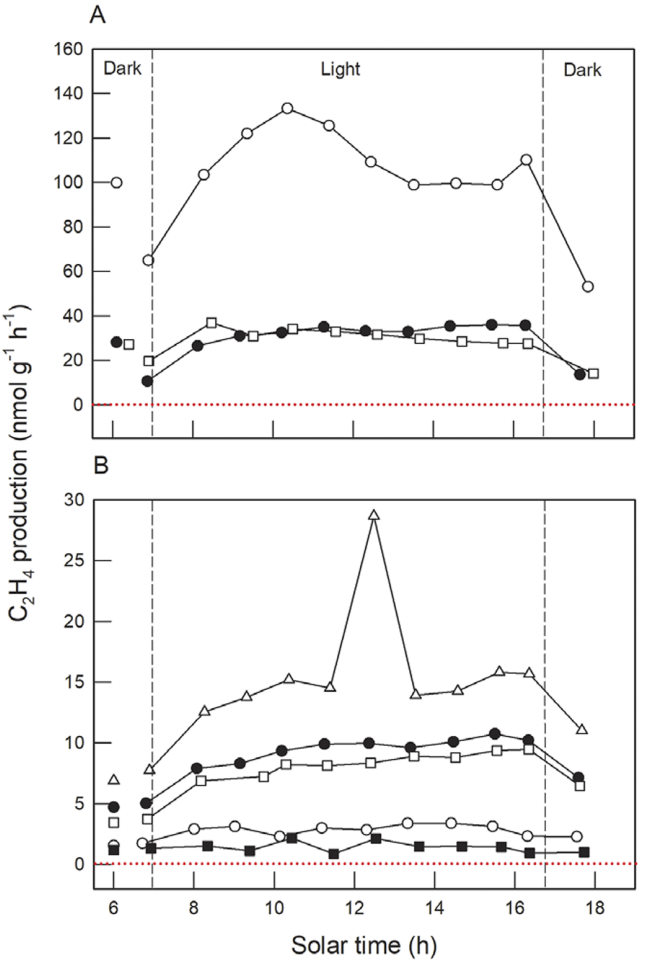


Figure 1. The progress of nitrogenase activity (acetylene reduction) in individual specimens of *Lichinella nigritella* (A) and *Thyrea confusa* (B) (November collections) during dark-light-dark transitions. Data points are plotted at the times at which incubations were terminated. Thallus temperatures and water contents were in the ranges 19–20 °C and 385–569% for *L. nigritella*, and 19–20 °C and 369–480% for *T. confusa.* High activity is recorded in the three specimens of *L. nigritella* and trace to low activity in the five specimens of *T. confusa* (see Table 1). Single points on the far left are activities recorded in preliminary assays conducted on the previous day. Note that the nitrogenase activity in the *T. confusa* specimen with the lowest activity is greater than zero (P = < 0.001, t-test). In colour online.

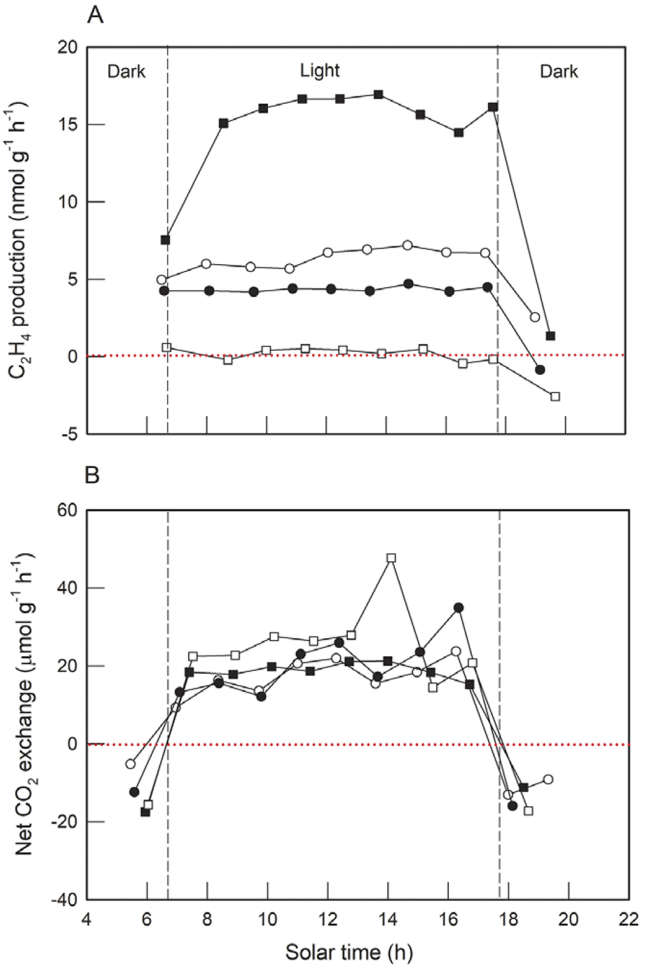


Figure 2. The progress of concurrent measurements of nitrogenase activity (acetylene reduction) (A) and net CO_2 exchange (B) during dark-light-dark transitions in four specimens of *Lichinella nigritella* collected in March. Points are plotted at the times at which incubations were terminated. Thallus temperatures and water contents were in the ranges 20–24 °C and 319–531%. Nitrogenase activities ranged from zero, trace to low (see Table 1). In colour online.

attempts to stimulate nitrogenase activity; these included thallus water content between 75–450% (see Fig. 4), the duration of hydration prior to conducting acetylene reduction assays (±added phosphate (10 µmol l^{-1} KH_2PO_4) in the bathing solution), and conducting assays during dark periods (Supplementary Material Tables S2–S4, available online).

All specimens tested, including those with zero or trace nitrogenase activity, were photosynthetically competent, achieving broadly comparable rates of net CO_2 exchange among replicates (Figs 2B, 3A & 4). Nonetheless, photosynthetic rates in *Lichinella* and *Thyrea* were low compared with those in *Peltigera membranacea* (Figs 3 & 4). There was some evidence, from an albeit limited data set, that variation in nitrogenase activity amongst these species was linked to photosynthetic capacity (Supplementary Material Fig. S1, available online).

In both *Lichinella* and *Thyrea*, nitrogenase activity was greater in the light, concurrent with photosynthesis, than in the dark (Figs 1–3). Amongst the limited data available, there was no evidence that rates of photosynthesis were lower in specimens with active nitrogenase. Transitions from dark to light and light to dark resulted in abrupt increases and decreases in nitrogenase activity; these changes were proportionately greater in specimens with high rates of activity and approached zero in those with low or trace activity (Figs 1, 2A, 3A & 5).

Of the four lichen species or species complexes for which ^{15}N natural abundance was measured, the non-N_2-fixing *Romjularia lurida* had the lowest $\delta^{15}N$ values and the highest thallus N concentrations (Table 2). However, $\delta^{15}N$ values in this species were markedly variable (range = −7.32 to −2.38‰) and the sample size was small. The established N_2-fixer, *Collema* spp. s. lat. (e.g. Kershaw & MacFarlane 1982) had a roughly similar $\delta^{15}N$ signature to that of the putative fixer *L. nigritella*, while *T. girardii* was the least ^{15}N-depleted and had the lowest N concentration.

Epiphytic free-living cyanobacteria occurred in varying quantities on the upper surfaces of thallus lobes of each of the three species, even after prior cleaning. The principal contaminant was referable to *Scytonema* (possibly *S. mirabilis*) while *Nostoc* and a

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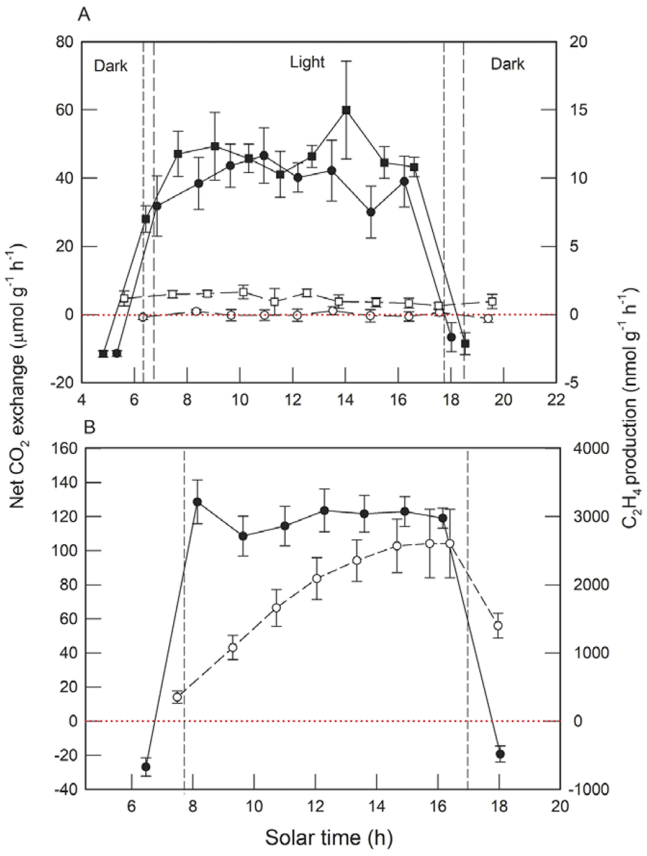


Figure 3. Progress of concurrent measurements of nitrogenase activity (acetylene reduction, open symbols) and net CO_2 exchange (solid symbols) in *Thyrea girardii* (circles = material collected in March, squares = material collected in September) (A) and *Peltigera membranacea* (B) during dark-light-dark transitions. Mean values are plotted ± 1 SE (n = 4 for *T. girardii*; n = 5–8 for *P. membranacea*) at time points at which incubations were terminated. Thallus temperatures and water contents for *T. girardii* were in the ranges 21–24 °C and 221–694% for March material, and 20–21 °C and 401–574% for September samples; values for *P. membranacea* were 18–22 °C and 283–322%. Nitrogenase activities in *T. girardii* were classed as trace (September) and zero (March) (see Table 1); note that nitrogenase activity in September was significantly greater than zero (P = < 0.001, t-test). In A, the vertical short and long dashed lines indicate the times of dark-light-dark transitions for March and September collections, respectively. In colour online.

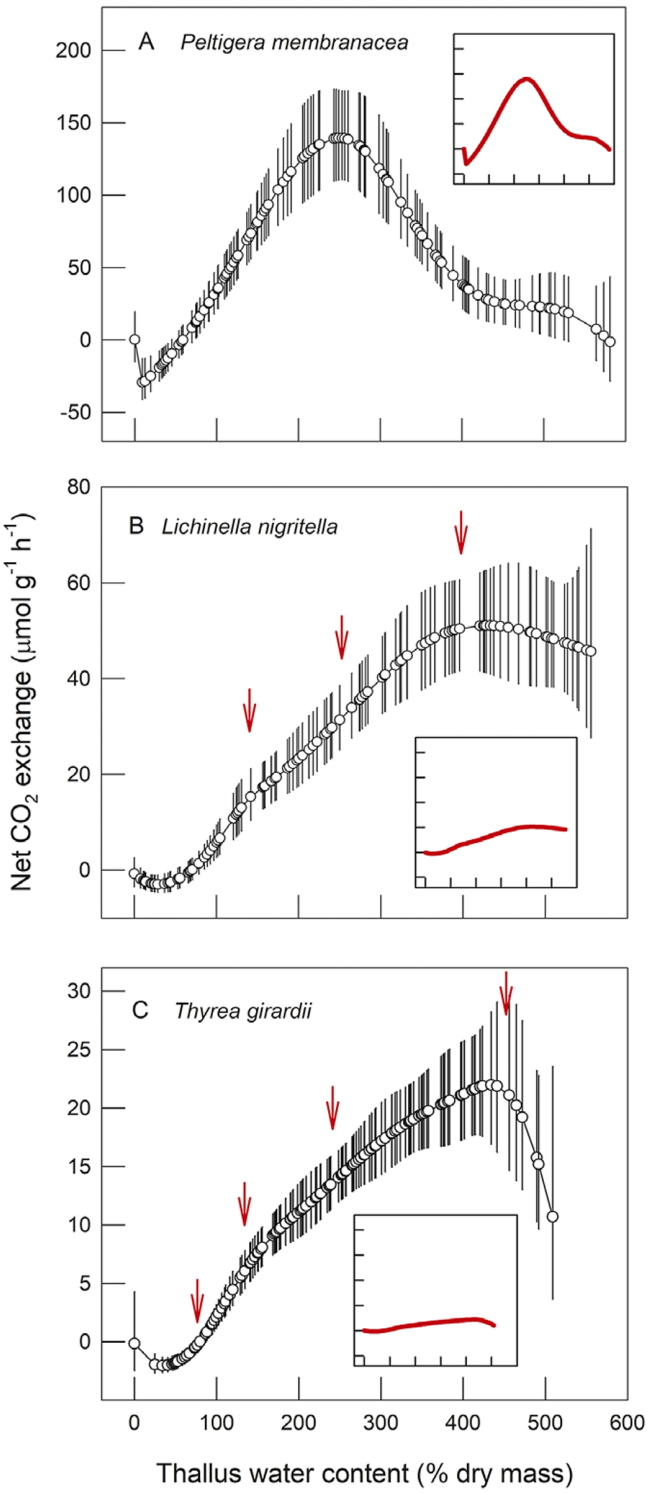


Figure 4. Relationship between thallus water content (% oven-dry mass) and net CO_2 exchange in *Peltigera membranacea* (A), *Lichinella nigritella* (B) and *Thyrea girardii* (C), measured at PFFR values of 170–200 μmol m^{-2} s^{-1} and thallus temperatures of 18–20, 20–21 and 22–26 °C in A, B and C, respectively. Inserts show these relationships drawn to the same scale as that for *P. membranacea*. Smooth curves are fitted together with 95% confidence intervals for each fitted value (n = 9, 8, and 8 for A, B and C, respectively). Drying times were 3–5 h. Arrows indicate thallus water content values at which nitrogenase assays were conducted as recorded in Supplementary Material Table S4 (available online). In colour online.

unicell referable to *Entophysalis* were also occasionally present in trace quantities (A. Pentecost, personal communication). Of the six specimens with high nitrogenase activities that were examined in detail, three had *Scytonema* filaments distributed across the upper surfaces of at least some lobes, while three had negligible contamination (Supplementary Material Table S5, available online). Of the six thalli with low activity, two had well-developed *Scytonema* populations on at least two lobes. On all other specimens, including all with zero or trace activity, surface contamination from epiphytic cyanobacteria was either sparse or absent. No heterocytous cyanobacteria were detected within thalli.

Discussion

Overview

Perhaps the most striking finding in this investigation was the sporadic nature of nitrogenase activity in the three species of *Lichinales* examined. This unpredictable activity presents a considerable problem for experimental studies on N_2 fixation in this lichen group with unicellular cyanobionts. These results are in stark contrast to experience with lichens containing heterocytous cyanobacteria in which nitrogenase activity is usually detectable within

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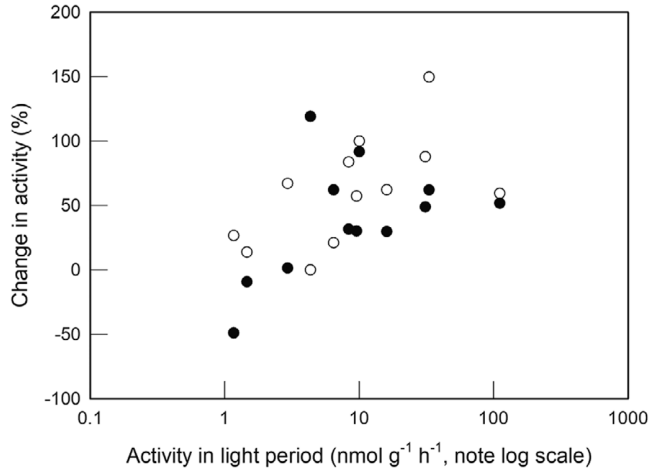


Figure 5. Relationship in *Lichinella nigritella*, *Thyrea confusa* and *T. girardii* between mean rate of nitrogenase activity (acetylene reduction) in the light (note log scale) and the percentage increase and decrease in activity, respectively, on transfer from dark to light (open circles) and light to dark (closed circles) (r = 0.51, P = 0.0101, bivariate Pearson correlation coefficient). Plotted values are derived from data in Figs 1, 2A and 3A. The most negative value reported in Fig. 2A was considered anomalous and omitted.

minutes of rehydrating freshly collected dry field material (Hitch & Stewart 1973; Huss-Danell 1977; Kershaw & Dzikowski 1977; Fritz-Sheridan 1988). The infrequency with which nitrogenase activity was detected in unicellular lichen cyanobionts is not without precedent. In our earlier report (Crittenden *et al.* 2007), we presented data for diel variation in nitrogenase activity in *Thyrea girardii*. However, *T. confusa* was likewise assayed in that study but no nitrogenase activity was recorded and, furthermore, of 12 replicate thalli of *T. girardii* prepared for assay, four had no activity. Snyder & Wullstein (1973) conducted acetylene reduction assays on *Peltula cylindrica* and *Pyrenopsis phaecocca*, species with unicellular cyanobionts, and were unable to detect activity that could be attributable to the cyanobacteria.

The identities of the cyanobacterial symbionts in the three lichen species studied here await critical assessment. Büdel & Henssen (1983) cultured photobionts from *Anema*, *Lichinella*, *Peccania* and *Psorotichia*, and considered that their developmental morphology was consistent with a *Chroococcidiopsis* sp. Later molecular studies have suggested that *Chroococcidiopsis* or other members of the *Chroococcidiopsidales* occur in several species of *Lichinella* and in *Thyrea pulvinata* (Jäger 2000 cited by Schultz & Büdel (2002); Fewer *et al.* 2002; Jung *et al.* 2021). Nonetheless, the phylogeny and taxonomy of genera in *Chroococcidiopsidales* remain unclear (Jung *et al.* 2024). Studies by Jung *et al.* (2021) on the identity and phylogeny of cyanobionts from the lichen genera *Gonohymenia*, *Lichinella*, *Peccania* and *Peltula* revealed an unexpected high cyanobiont diversity, with all being described as new species: five were new unicellular species, two of which being assigned to new genera, and two (from *Gonohymenia* sp. and *Lichinella cribellifera*) were determined as new species of *Komarekiella*, a genus of heterocytous cyanobacteria. The authors found that none of these new species was able to grow on an N-free medium. Rippka *et al.* (1979) reported that *Chroococcidiopsis* expresses nitrogenase activity only under anaerobic conditions, while it should be noted that cultures were maintained and assayed (see Rippka & Waterbury 1977) under continuous illumination (cf. Boison *et al.* 2004). By contrast, several authors have measured aerobic nitrogenase activity in free-living endolithic *Chroococcidiopsis*-type unicellular cyanobacterial

Table 2. ^{15}N natural abundance (δ^{15}N) and total thallus N concentration in four lichen species co-occurring on limestone cliffs in the study area. Values assigned the same letters are not significant at the P = 0.05 level (one-way ANOVA).

Species	δ^{15}N (‰)	N (%)	*n*
Thyrea girardii	−0.84 ± 0.31[a]	2.57 ± 0.08[a]	10
Lichinella nigritella	−3.41 ± 0.46[b]	4.28 ± 0.08[b]	10
Collema spp. s. lat.*	−2.56 ± 0.11[a,b]	4.45 ± 0.27[b]	9
Romjularia lurida	−5.36 ± 1.01[b]	6.15 ± 0.25[b]	5

*See Table 1

populations, although interference from heterocytous species could not be ruled out (Friedmann & Kibler 1980; Boison *et al.* 2004; Banerjee & Verma 2009). Friedmann & Kibler (1980) also commented on the low frequency with which nitrogenase activity could be detected in endolithic samples with, for example, activity being recorded in only one of 11 rock samples from the Antarctic Dry Valleys containing endolithic cyanobacteria, purportedly unicells. This infrequency with which nitrogenase activity is detected in free-living *Chroococcidiopsis*-type unicells has led to uncertainty as to whether lichens with unicellular cyanobacteria are likely to be diazotrophic.

The values of δ^{15}N measured in the four lichen species/genera growing on limestone cliffs (Table 2) are broadly in accord with published data. Crittenden *et al.* (2023) compared δ^{15}N values amongst eight species of lichen containing heterocytous cyanobacteria and with contrasting N_2-fixing capacities; mean values varied from −4.3‰ to +2.6‰, a range that accommodates values for *Lichinella* and *Thyrea* reported here. The eight values were weakly and non-significantly positively related to maximum rates of nitrogenase activity. (Note that according to Zhang *et al.* (2014) the theoretical δ^{15}N value for ammonium produced by nitrogenase is *c.* −1.0‰ and that Dojani *et al.* (2007) reported a value of −1.9 ± 0.9‰ (n = 15) for cyanobacterial biofilms, containing both unicellular and heterocytous species, on an inselberg in French Guiana.) Chlorolichens generally have δ^{15}N values below this range (Russow *et al.* 2005; Beck & Mayr 2012; Hurtado *et al.* 2020; Petix *et al.* 2024 (and references therein), 2025). Nevertheless, separation between N_2-fixing and non-N_2-fixing lichens based on δ^{15}N values is not complete. δ^{15}N values for chlorolichens that fall within the range for N_2-fixers have been reported for species on a rocky seashore (Riera 2005), for *Siphula ceratites* on peatland (Asada *et al.* 2005) and for eutrophication tolerant species such as *Parmelia saxatilis* (Crittenden *et al.* 2023) and *Gallowayella borealis* (= *Xanthomendoza borealis*) (Crittenden *et al.* 2015) amongst others. Furthermore, the above interpretations are based on a limited range of lichens for which δ^{15}N values are currently available. Accordingly, the δ^{15}N measurements for *Lichinella* and *Thyrea*, while being comparable with those of other N_2-fixing lichens, need to be considered with caution as evidence of diazotrophy. In addition, such an interpretation would need to consider how relatively high δ^{15}N values could be generated by only occasional episodes of nitrogenase activity.

Potential confounding factors

Two factors could conceivably present pitfalls in the interpretation of the present results. First, the *Thyrea* and *Lichinella* specimens studied were 'contaminated' to varying degrees with surface-bound

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epiphytic heterocytous cyanobacteria, principally *Scytonema*. Even though the quantity of this 'extramatricullar' material was minute compared with that of the unicellular cyanobiont, it raises the question as to whether nitrogenase activity in this heterocytous component was detectable and could have significantly contributed to nitrogenase activity in at least some specimens. Guidance on this question might be provided by the results of Fritz-Sheridan (1987) who conducted *in situ* acetylene reduction assays on *Scytonema* mats and on adjacent stands of the N_2-fixing lichen *Stereocaulon virgatum* in Guadeloupe. On a unit dry mass basis, nitrogenase activities in well-hydrated cyanobacterial mats and lichen thalli were remarkably similar: between 53.7 ± 18.2 and 87.2 ± 19.2 nmol C_2H_4 g^{-1} h^{-1} in *Scytonema* mats, and 52.9 ± 27.2 and 74.0 ± 21.0 nmol C_2H_4 g^{-1} h^{-1} in *S. virgatum*. It seems probable, then, that at the very least several milligrams of epiphytic *Scytonema* would be required per thallus in order for its nitrogenase activity to be detected, whereas the observed 'contamination' probably amounted to several micrograms at most. B. Büdel (personal communication) has observed *Scytonema* growing epiphytically quite widely among the *Lichinaceae* and *Peltulaceae* and has reached a similar conclusion regarding its potential acetylene reduction signal. Nitrogenase activity in heterocytous cyanobacteria, and lichens in which they occur, are highly responsive to dark to light and light to dark transitions, showing abrupt increases and decreases, respectively, in nitrogenase activity both under laboratory (MacFarlane *et al.* 1976; Bottomley & Stewart 1977; Huss-Danell 1979; Mullineaux *et al.* 1981; Ernst *et al.* 1990) and field conditions (Crittenden & Kershaw1979; Coxson & Kershaw 1983; Quesada *et al.* 1998). The responses to such transitions in *Lichinella* and *Thyrea* were positively correlated with the mean level of activity in the light (Fig. 5), with trace activity appearing unresponsive. If the source of trace activity was a small population of highly active heterocytous epiphytes, then a response to these abrupt changes in illumination would probably have been evident. The absence of such a response is more consistent with a larger population of photobiont cells with activity that is sufficiently low to be sustained by cellular reserves and/or low rates of C-transfer from the mycobiont. Taking these findings together, it seems doubtful that nitrogenase activity recorded in *Lichinella* and *Thyrea* is owing to contamination. Furthermore, the apparent link between rates of nitrogenase activity and photosynthesis amongst *Lichinella*, *Peltigera* and *Thyrea* (Supplementary Material Fig. S1, available online), if real, would seem unlikely to be generated by contamination.

Second, N pollution also needs to be considered as a potential source of interference. Rates of atmospheric N deposition in Catalonia are the highest in Spain (García-Gómez *et al.* 2014; Vivanco *et al.* 2018). Modelled values of mean annual total inorganic N deposition among the four lichen collection sites ranged from 14–19 kg ha^{-1} in 2017 (downloaded from https://www.emep.int/mscw/mscw_moddata.html). Combined N is an antagonist of N_2 fixation. Crittenden *et al.* (2023) reported that in the British Isles the tripartite montane lichen *Stereocaulon vesuvianum* does not produce cephalodia, and does not fix N_2, at mean annual N deposition rates > 10 kg ha^{-1}, with the most significant independent variable predicting loss of cephalodia being dry deposition of reduced N (NH_y). The authors speculated that the low rates of N income from N_2 fixation in *S. vesuvianum* (acetylene reduction rate 20–70 nmol C_2H_4 g^{-1} h^{-1}) could perhaps be readily supplanted by combined N income from elevated N deposition. Rates of nitrogenase activity in *Lichinella* and *Thyrea* (≤ *c.* 100 nmol C_2H_4 g^{-1} h^{-1}) are similar to those in *S. vesuvianum* and a negative effect of N enrichment might also have occurred in these species. However, the oceanic climate of montane western Britain (the principal range of British populations of *S. vesuvianum*) contrasts strongly with the Mediterranean climate of the Barcelona region where both annual rainfall and the number of days with rain are considerably lower (by approximately an order of magnitude and a factor of 4, respectively), and the evaporating power of the atmosphere is higher (annual mean saturation deficit is greater by a factor of 3). Dry deposition of ammonia to lichens is likely to be lower in a substantially drier climate. A more detailed and quantitative discussion of this topic is presented in Supplementary Material Note S1 (available online).

On the infrequency of nitrogenase activity in Lichinella *and* Thyrea

The sporadic nature of nitrogenase activity in *Lichinella* and *Thyrea* might indicate that the availability of combined forms of N in their habitats is only occasionally insufficient to meet their growth-led demand. Unicellular cyanobacteria are well placed to switch from oxygenic photosynthesis to N_2 fixation for short periods of time. As is the case for many species in the *Lichinaceae*, the habitats from which specimens were collected for the present work were relatively extreme: exposed, near-vertical cliff faces experiencing long periods of continuous desiccation, high insolation and high temperatures. Accordingly, *Thyrea* and *Lichinella* probably have low annual growth rates and moderate growth-led demand for N. The low photosynthetic rates in *L. nigritella* and *T. girardii* (Fig. 4) are in line with this suggestion, although we are mindful that the data sets for net CO_2 exchange presented here are unifactorial and do not comply with the multifactorial approach advocated by Kershaw (1985). Nutrients are delivered to these lichens in dry deposits and rainfall that are intercepted directly by thalli and, in due course, in both descending surface run-off and seepage water. The presence of *Rusavskia elegans* (= *Xanthoria elegans*) in the most well-marked seepage tracks on some cliffs sampled perhaps indicates that seepage water in these locations is nutrient enriched.

Lichens that achieve high rates of N_2 fixation (with rates of acetylene reduction > 1 μmol C_2H_4 g^{-1} h^{-1}) have, with few exceptions, *Nostoc* as either the primary or secondary photobiont (cf. Sanders & Masumoto 2021). These include species of *Collema* s. lat. (Kershaw & MacFarlane 1982), *Leptogium* and *Pectenia* (=*Degelia*) (Hitch & Millbank 1975), *Lobaria* (Alexander *et al.* 1974), *Nephroma* and *Solorina* (Kallio *et al.* 1972; Granhall & Selander 1973), *Peltigera* (Hitch & Stewart 1973), and *Pseudocyphellaria* and *Sticta* (Green *et al.* 1980). Notable exceptions are *Lichina* (Hitch & Stewart 1973), in which the principal cyanobiont is *Rivularia* (Ortiz-Álvarez *et al.* 2015), and the basidiolichens *Dictyonema* (Fritz-Sheridan 1988) and *Cora* (Fritz-Sheridan & Portécop 1987), containing the cyanobacterium *Rhizonema* (Lücking *et al.* 2014). This group of lichens with high rates of activity is probably adapted to habitats in which their growth-led demands for N greatly exceed the availability of combined forms of N in, for example, atmospheric deposition and canopy throughfall. Therefore, in these circumstances, constant maintenance of N_2-fixing capacity, and a metabolically costly heterocyte population, is advantageous. Lichens with lower N_2-fixing capacities (e.g. with maximum rates of nitrogenase activity < 1 μmol C_2H_4 g^{-1} h^{-1}) presumably have lower growth-led N demands yet perhaps occupy habitats where this demand is still not met by income of combined N. This would include species of *Stereocaulon* (Crittenden 1975), *Ephebe* and *Placynthium* (Hitch & Millbank 1975), and *Placopsis* (Crittenden 1975; Raggio *et al.* 2012), containing the cyanobionts *Stigonema*, *Dichothrix* and *Tolypothrix*

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(Sanders & Masumoto 2021). These lichens occupy open habitats where they intercept atmospheric deposition directly with minimum modification, and potential nutrient enrichment, by overlying plant canopies. It is tempting to speculate that cyanobiont species other than *Nostoc* may be better suited to achieving more modest rates of N_2 fixation. Regrettably there is a dearth of comparative data on rates of nitrogenase activity achieved by different species and genera of cyanobacteria.

While lichens with unicellular cyanobionts can in theory readily switch between N_2-fixing and non-N_2-fixing states, those with heterocytous cyanobionts may be less capable of such rapid adjustment. In free-living cyanobacteria, both heterocytous and non-heterocytous species, nitrogenase activity and synthesis is inhibited by combined N (Fogg *et al.* 1973; Stewart 1973). Inhibition can occur within several hours of exposure; examples include the almost complete loss of nitrogenase activity in *Anabaena* sp. (Bottomley *et al.* 1979), *Plectonema boryanum* (=*Leptolynbya boryana*) (Rai *et al.* 1992), *Gloeothece* sp. (Cheng *et al.* 1999) and *Crocosphaera watsonii* (Dekaezemacker & Bonnet 2011) within 2–12 h in response to NH_4^+ (10, 2, 2 and 0.01 mM solutions, respectively). There is also evidence that exposure to combined N inhibits nitrogenase activity in lichenized cyanobacteria (Kytöviita & Crittenden (1994) and references therein). Sustained exposure of free-living cyanobacteria to combined N also inhibits heterocyte formation. For example, Willis *et al.* (2016) report that when axenic cultures of *Cylindrospermopsis raciborskii*, a species with a single terminal heterocyte on each trichome, grown in N-free medium were transferred to an N-replete medium (containing 0.9 mM NO_3^-), heterocyte abundance declined from one per trichome to zero over a period of 20 days, some heterocytes undergoing degradation and many apparently being shed. Conversely, transferring the cyanobacterium from an N-replete to N-free medium resulted in an increase in heterocyte frequency from zero to one per trichome over a similar time period. Likewise, Chua *et al.* (2012) found that *Nostoc* isolated from *Pseudocyphelaria crocata* and cultured axenically in an N-replete medium (containing 2.5 mM NH_4^+ + 3.0 mM NO_3^-) produced heterocytes within 24 h on transfer to an N-free medium. It is not known at what rate and where heterocyte frequency could be adjusted within a lichen thallus if exposed to sustained N enrichment or deprivation. Heterocytes first develop in the marginal and apical growing regions of lichen thalli (Bergman & Hällbom 1982; Janson *et al.* 1993; Chua *et al.* 2012) and hence changes in the frequency of heterocyte differentiation here will determine heterocyte abundance in new photosynthetic tissue. It is less clear how heterocytous cyanobionts in mature, fully differentiated regions of thalli would respond to changes in N supply. Heterocytes are terminally determinate structures and cannot re-differentiate back to vegetative cells (Fogg *et al.* 1973; Zeng & Zhang 2022). Therefore, a reduction in heterocyte numbers in extant photosynthetic tissue in response to N enrichment, if indeed this occurs, would likely involve heterocyte degeneration. Here also the vegetative cells of cyanobionts exist as enlarged or 'outsized' cells thought to be associated with cessation of cell division (Bergman & Hällbom 1982; Hill 1984, 1989; Honegger 1993; Chua *et al.* 2012), arguably creating a quiescent cell factory with elevated photosynthetic capacity (cf. Garfias-Gallegos *et al.* 2025). This leads to the thought that development of new heterocytes in response to N deprivation would probably require the recovery of cell potency. These conundrums largely relate to bipartite cyanolichens. The size and abundance of cephalodia in tripartite lichens have been shown to decline in response to N enrichment (Palmqvist *et al.* 2017; Crittenden *et al.* 2023). Crittenden *et al.* (2023) speculated that the development of cephalodia in N-enriched acephalodiate populations of *Stereocaulon vesuvianum* could take several years following reduction of N deposition rates. These arguments suggest that lichens with heterocytous cyanobionts are perhaps less suited than those with unicellular cyanobionts to environments in which N supply is only occasionally limiting.

Lichinella *and* Thyrea *fix nitrogen in the light*

Another notable finding in this study is that maximum rates of nitrogenase activity in *Lichinella* and *Thyrea* occur in the light. While this largely confirms the results of an earlier study (Crittenden *et al.* 2007), here we further show that thalli with nitrogenase activity also assimilate CO_2 (Figs 2 & 3A). This contrasts with natural populations of free-living unicellular cyanobacteria that fix N_2 during dark periods, providing temporal separation from oxygenic photosynthesis in the light (Griffiths *et al.* 1987; Wilson *et al.* 2017). Nonetheless, laboratory cultures of the unicells *Gloeothece* and *Crocosphaera* can be induced to fix N_2 in the light when grown in continuous illumination (Gallon *et al.* 1974; Grobbelaar *et al.* 1986; Du & Gallon 1993; Schneegurt *et al.* 1994; Taniuchi *et al.* 2008), in well-aerated continuous cultures or under a low light/dark regime (e.g. with PPFR values of 5–10 μmol m^{-2} s^{-1}) (Ortega-Calvo & Stal 1991).

In free-living unicellular cyanobacteria, N_2 fixation in the dark is fuelled by the utilization of carbohydrate reserves. Most frequently these are glycogen or related α-glucans, the principal C-storage products in cyanobacteria, often discernible as granules by electron microscopy (Fay 1983). Carbon storage granules form during photosynthesis in the light and are then catabolized in support of nitrogenase activity in the dark in a diel oscillation (Mullineaux *et al.* 1980; Livingstone *et al.* 1984; Reade *et al.* 1999; Gallon 2001; Mohr *et al.* 2010; Aryal *et al.* 2011; Červený *et al.* 2013; Welkie *et al.* 2013; Liberton *et al.* 2019). It is perhaps noteworthy then that Paran *et al.* (1971) did not observe glucan/glycogen granules in the unicellular cyanobiont of *Lichinella sinaica* (= *Gonohymenia sinaica*) and they speculated that this absence might result from a rapid flow of photosynthate to the mycobiont. This suggestion is in line with the possibility that in *Lichinella* and *Thyrea* respiratory substrate for nitrogenase activity is supplied by the fungus rather than reserves within the cyanobiont. Photosynthate derivatives in the mycelium of the photobiont layer are likely to be in most plentiful supply in the light before their concentration is depleted by translocation to sink regions in the thallus, such as zones of growth and surface tissues (Tapper 1981); accordingly, this could be a principal reason why lichenized unicellular cyanobacteria fix N_2 in the light, a period when the supply of respiratory substrate from the mycobiont is maximal. While this may seem plausible, it is also possible that the specimens examined by Paran *et al.* had become desiccated at a point in the diel cycle before storage granules had re-formed. In free-living heterocytous cyanobacteria, C-storage granules and reserves undergo diel cycles of production and depletion as reported in unicellular species (Ernst *et al.* 1990) and they have been found frequently in heterocytous lichen cyanobionts (Boissière 1972, 1982; Rai *et al.* 1981; Bergman & Hällbom 1982; Bergman & Huss-Danell 1983; Bergman & Rai 1989), although these observations are not without exception (Peat 1968; Bousfield & Peat 1976). Boissière (1982) reported that if *Peltigera canina* was maintained in the dark prior to chemical fixation, the polyglucoside granules observed previously in the *Nostoc* photobiont then partially disappeared. Boissière (1982) also noted in *P. canina* that granules were abundant in

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young *Nostoc* cells but generally lacking in fully developed and old cells. Additional work is required to verify the absence (or otherwise) of C-storage granules in unicellular lichen cyanobionts, a condition that would be in accord with N_2 fixation being fuelled by the mycobiont.

On the location of nitrogenase activity in thalli

Where in thalli of *Lichinella* and *Thyrea* N_2 fixation occurs remains to be determined. Cyanobiont cells that switch from photosynthesis to N_2 fixation could be dispersed throughout the photobiont population, a suggestion that finds support from studies on free-living planktonic unicells. *Crocosphaera watsonii* has been found to form loose colonies or aggregations of between 3–250 cells both in natural planktonic populations (Foster *et al.* (2013) and references therein) and in laboratory cultures (Masuda *et al.* 2020). Using nanoscale secondary ion mass spectrometry (nanoSIMS), these authors demonstrated that only a subset of cells in an aggregate (34–75%) fixed N_2 (cf. Mohr *et al.* 2013) while assimilating little CO_2, a phenomenon that had earlier been predicted to occur in *Gloeothece* by Stal and co-workers (Ortega-Calvo & Stal 1991; Compaoré & Stal 2010). Additionally, Dron *et al.* (2012) estimated that 26–60% of N_2 fixed by *Crocosphaera* might be released into the environment. Masuda *et al.* (2020) speculated that this released N could be available to non-diazotrophic cells and that such heterogeneity in N_2-fixing capacity within an aggregate would maximize C-use efficiency. The authors constructed a simulation model to predict the effects of several factors on C costs of diazotrophy in *C. watsoniia*. One such factor was the efficiency with which released N could be taken up by neighbouring non-N_2-fixing cells. In a lichen thallus this will approach 100% since current understanding is that fixed N is largely transferred to the mycobiont (Nash 2008), reducing the C cost of N_2 fixation. Note that the possibility of functional heterogeneity amongst photobiont cells had been raised previously by Boissière (1987), whereby some vegetative cells of *Nostoc* in *Peltigera* might be partially disengaged from C transfer to the mycobiont. Gallon (2001) and Ortega-Calvo & Stal (1991) proposed that N_2 fixation and photosynthesis in *Gloeothece* could occur in the same cell if the rate of photosynthetic O_2 production is sufficiently low for it to be consumed by respiratory protection, a suggestion that has subsequently found support in the results of studies on *Crocosphaera* (Großkopf & LaRoche 2012; Rabouille *et al.* 2021; Bandyopadhyay *et al.* 2024). In addition, there might be microsites within lichen thalli where N_2 fixation is favoured, such as areas of self-shading below overlapping lobes where operational PPFR values could be low enough to promote nitrogenase activity in the light (cf. Ortega-Calvo & Stal 1991); hydrated single lobes of *T. girardii*, for example, transmit *c.* 5% of incident light (Supplementary Material Note S2, available online).

Finally, in free-living populations of unicellular cyanobacteria, the cyclic switching from oxygenic photosynthesis in the light to N_2 fixation in the dark is coupled to, or at least parallels, the cell cycle. In *Crocosphaera*, in both laboratory cultures (Mitsui *et al.* 1986; Mohr *et al.* 2010; Shi *et al.* 2010; Dron *et al.* 2012, 2013) and in natural planktonic populations (Wilson *et al.* 2017), and also in cultures of *Gloeothece* (Gallon *et al.* 1974), photosynthesis is maximal immediately prior to cell division while nitrogenase activity develops post division. Proposing a link between nitrogenase activity and the cell cycle in lichenized unicellular cyanobacteria, in which the cell cycle has largely become arrested, could be problematic. However, we have noted that in *T. girardii* small photobiont cells, presumably the products of recent division, are not confined to the thallus margins but can be seen throughout the photobiont layer. Hence an ontogenetic basis for heterogeneity in N_2-fixing activity within a unicellular cyanobiont population in a lichen thallus might also be at play.

Concluding comment

This report possibly raises as many, if not more, questions than it answers. However, some consolation might be taken from the large body of work pertaining to *Trichodesmium*, a genus of marine diazotrophic filamentous non-heterocytous cyanobacteria considered keystone species in pelagic N cycles (Zehr & Kudela 2011). As is the case with *Lichinella* and *Thyrea*, *Trichodesmium* spp. fix N_2 in the daytime concurrent with photosynthesis, yet after decades of research the mechanisms by which this is achieved, according to Zehr & Capone (2024), remain elusive.

More work is needed to better understand the strategies by which N_2 fixation is achieved in lichens with unicellular cyanobionts. Above all, perhaps, is how nitrogenase activity is distributed within thalli. Further research might be most effective using lichen populations that are near to research facilities, and which can be accessed and sampled frequently (e.g. on a daily basis). Such populations should preferably be in a region with low rates of N deposition (e.g. < 10 kg N ha^{-1} y^{-1}, cf. Crittenden *et al.* (2023)).

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