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The occurrence of the chloroplast pyrenoid is correlated with the activity of a CO_2-concentrating mechanism and carbon isotope discrimination in lichens and bryophytes

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Abstract. The organic-matter carbon isotope discrimination (Δ) of lichens with a wide range of photobiont and/or cyanobiont associations was used to determine the presence or absence of a carbon-concentrating mechanism (CCM). Two groups were identified within the lichens with green algal photobionts. One group was characterised by low, more C_4-like Δ values ($\Delta < 15‰$), the other by higher, more C_3-like Δ values ($\Delta > 18‰$). Tri-partite lichens (lichens with a green alga as the primary photobiont and cyanobacteria within internal or external cephalodia) occurred in both groups. All lichens with cyanobacterial photobionts had low Δ values ($\Delta < 15‰$). The activity of the CCM, organic-matter Δ values, on-line Δ values and gas-exchange characteristics correlated with the presence of a pyrenoid in the algal chloroplast. Consistent with previous findings, lichens with *Trebouxia* as the primary photobiont possessed an active CCM while those containing *Coccomyxa* did not. Organic Δ values for lichens with *Stichococcus* as the photobiont varied between 11 and 28‰. The lichen genera *Endocarpon* and *Dermatocarpon* (*Stichococcus* + pyrenoid) had C_4-like organic Δ values (Δ = 11 to 16.5‰) whereas the genus *Chaenotheca* (*Stichococcus* − pyrenoid) was characterised by high C_3-like Δ values (Δ = 22 to 28‰), unless it associated with *Trebouxia* (Δ = 16‰). Gas-exchange measurements demonstrated that *Dermatocarpon* had an affinity for CO_2 comparable to those species which possessed the CCM, with $K_{0.5}$ = 200–215 $\mu l \cdot l^{-1}$, compensation point (Γ) = 45–48 $\mu l \cdot l^{-1}$, compared with $K_{0.5}$ = 195 $\mu l \cdot l^{-1}$, Γ = 44 $\mu l \cdot l^{-1}$ for Trebouxioid lichens. Furthermore, lichens with *Stichococcus* as their photobiont released a small pool (24.2 ± 1.9 to 34.2 ± 2.5 nmol · mg^{-1} Chl) of inorganic carbon similar to that released by Trebouxioid lichens [CCM present, dissolved inorganic carbon (DIC) pool size = 51.0 ± 2.8 nmol · mg^{-1} Chl]. Lichens with *Trentepohlia* as photobiont did not possess an active CCM, with high C_3-like organic Δ values (Δ = 18‰ to 23‰). In particular, *Roccella phycopsis* had very high on-line Δ values (Δ = 30 to 33‰), a low affinity for CO_2 ($K_{0.5}$ = 400 $\mu l \cdot l^{-1}$, Γ = 120 $\mu l \cdot l^{-1}$) and a negligible DIC pool. These responses were comparable to those from lichens with *Coccomyxa* as the primary photobiont with *Nostoc* in cephalodia (organic Δ = 17 to 25‰, on-line Δ = 16 to 21‰, $K_{0.5}$ = 388 $\mu l \cdot l^{-1}$, Γ = 85 $\mu l \cdot l^{-1}$, DIC pool size = 8.5 ± 2.4 nmol · mg^{-1} Chl). The relative importance of refixation of respiratory CO_2 and variations in source isotope signature were considered to account for any variation between on-line and organic Δ. Organic Δ was also measured for species of Anthocerotae and Hepaticae which contain pyrenoids and/or *Nostoc* enclosed within the thallus. The results of this screening showed that the pyrenoid is correlated with low, more C_4-like organic Δ values (Δ = 7 to 12‰ for members of the Anthocerotae with a pyrenoid compared with Δ = 17 to 28‰ for the Hepaticae with and without *Nostoc* in vesicles) and confirms that the pyrenoid plays a fundamental role in the functioning of the CCM in microalgal photobionts and some bryophytes.

Key words: Anthocerotae – Cyanobacterium – Microalga – Photobiont – Photosynthesis

Introduction

A lichen is a symbiotic association between a fungus (the mycobiont) and a photosynthetic microalga or cyanobacterium (the photobiont). In some lichens, the tripartite lichens, the primary photobiont is a microalga and a secondary photobiont, a cyanobacterium, is present within cephalodia, structures which may be located externally or internally within the thallus. The majority of mycobionts form lichenized associations with only one algal genera

Abbreviations and symbols: CCM = carbon-concentrating mechanism; DIC = dissolved inorganic carbon ($CO_2 + HCO_3^- + CO_3^{2-}$); DW = dry weight; $K_{0.5}$ = external concentration of CO_2 at which half-maximal rates of CO_2 assimilation are reached; photobiont = photosynthetic organism present in the lichen; Rubisco = ribulose-1,5-bisphosphate carboxylase-oxygenase; Δ = carbon isotope discrimination (‰); $\delta^{13}C$ = carbon isotope ratio (‰).

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although a few mycobionts have been shown to associate with more than one photobiont (e.g. *Psilolechia lucida* and *Chaenotheca chlorella* associate with either *Trebouxia* or *Stichococcus*). In terms of lichen ecophysiology, the interactions between environmental effects and photosynthetic physiology in determining net rates of carbon fixation during photosynthesis have recently been evaluated (Lange and Ziegler 1986; Lange 1988; Badger and Price 1992; Lange et al. 1994; Palmqvist et al. 1994a, b).

Within the lichen thallus the photobiont cells are enclosed in a matrix of fungal hyphae and must absorb water before photosynthesis can be activated. The effect of wetting the thallus is to cause the fungal hyphae to swell and, depending upon the degree of wetting, the diffusion of CO_2 through the fungal matrix may be impeded and net assimilation rates decline (Lange 1980; Cowan et al. 1992). Recently, species have been identified where net assimilation rates are maintained even at high thallus water contents (Lange et al. 1994). Theoretically, diffusion resistance to the passage of CO_2 through the thallus to the carboxylation site may be demonstrated by a lower thallus carbon isotope ratio (less-negative $\delta^{13}C$ values; Lange and Ziegler 1986). However it has also been shown that cyanobacteria and many microalgae, either free-living or in lichen symbioses, possess a carbon-concentrating mechanism (CCM) which could also confer low carbon isotope discrimination (Δ) on the thallus (Raven et al. 1990; Máguas et al. 1993, 1995).

Recent studies of photosynthesis in lichens have shown that the characteristics of the photobiont partners in symbioses reflect those of the free-living counterparts (Máguas et al. 1993; Palmqvist et al. 1994a). Thus consideration of the photosynthetic physiology of free-living cyanobacteria and microalgae may provide important insights into carbon accumulation mechanisms in lichens.

Cyanobacteria. All cyanobacteria possess a biophysical CCM which serves to raise the concentration of CO_2 in carboxysomes, where ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is located (Raven 1985; Raven et al. 1990; Kaplan et al. 1991; Badger and Price 1992). This is necessary since the cyanobacterial Rubisco has an inherently low affinity for CO_2 (Coleman 1991; Badger and Price 1992) and the increased concentration of CO_2 at the site of the Rubisco also diminishes the photorespiratory activity of the cyanobacterium. Gas-exchange studies and measurements of Δ have linked the high affinity for CO_2 of both free-living and lichenized *Nostoc* with the activity of a CCM (Palmqvist 1993; Palmqvist et al. 1994b).

Carbon isotope discrimination (Δ) represents the $\delta^{13}C$ value corrected for source CO_2 isotope composition (Farquhar et al. 1989) for both organic matter and samples collected instantaneously during photosynthesis. For cyanobacterial lichens, Δ was in the range 10 to 15‰, close to values from higher plants exhibiting C_4 photosynthesis (Máguas et al. 1993; Palmqvist et al. 1994a). Evidence for the presence of an intracellular pool of CO_2 is provided by the transient release of CO_2 when a photosynthetically active cyanobacterial lichen is darkened (Badger et al. 1993). As well as being the primary photobiont partner of many species of lichen, cyanobacteria occur in internal or external cephalodia in many lichens (James and Henssen 1976). In addition to lichen symbioses, *Nostoc* also occurs in vesicles within the thallus in some genera of Hepaticae (*Blasia pusilla*) and Anthocerotae (*Anthoceros* spp.).

Microalgae. Many microalgal species also possess a CCM (Beardall et al. 1982; Coleman 1991; Badger and Price 1992; Palmqvist et al. 1994c; Raven 1991), although the precise mechanism is still under investigation. The pyrenoid, a protein-rich organelle sheathed with starch and containing high concentrations of Rubisco is thought to play a fundamental role in the operation of the CCM, particularly at low external levels of CO_2 (Kuchitsu et al. 1991; Osafune et al. 1992; Pronina and Semenenko 1992; Ramazanov et al. 1993). Pyrenoids are restricted in their occurrence to some photobiont species of microalgae including *Trebouxia*, the commonest green algal genus found in lichen symbioses, and *Nanochloris*, *Chlorella and Pseudochlorella*, less frequently occurring photobionts. A number of species of the algal genus *Stichococcus* occur as photobionts with some species of this genus possessing pyrenoids (Ahmadijian and Heikilla 1970) and a CCM (Hogetsu and Miyachi 1977; Munoz and Merrett 1988). Pyrenoids are also found in a range of green macroalgae. Elsewhere in the plant kingdom pyrenoids occur only in the Anthocerotae. The Hepaticae and Musci do not possess these structures, nor do any higher plants. Lichens with the green alga *Trebouxia* as photobiont accumulate internal pools of CO_2 although the pool sizes are considerably lower than those of the cyanobacterial lichens (Badger et al. 1993), whereas in *Coccomyxa*, whether free-living or lichenized the CCM is absent (Palmqvist 1993; Palmqvist et al. 1994c).

Aims. In order to re-evaluate the photosynthetic physiology of lichens in terms of the occurrence of a pyrenoid, in this paper we survey Δ in organic material for green algal, tri-partite and cyanobacterial lichens which possess a wide range of photobiont genera, as compared with several species of bryophyte. We have employed two recently developed techniques, viz., on-line Δ and direct measurement of the pool of dissolved inorganic carbon (DIC), to identify the presence of a CCM. Measurements of instantaneous on-line Δ across a range of water contents have allowed us to quantify the effects of diffusion limitation and evaluate the contribution to thallus organic Δ. We intend to clarify photobiont groups in relation to the activity of a CCM within lichens, (see also Máguas et al. 1993, 1995) and to establish the correlation between the activity of a CCM and the presence of a pyrenoid in the algal chloroplast for lichens and bryophytes.

Materials and methods

Choice of material and acclimation procedure

Organic-matter Δ. An initial screening of thallus organic Δ was carried out on 58 species of lichens and bryophytes. Samples were gathered fresh from field sites in Britain, Norway and France or were supplied from herbarium collections.

On-line Δ, gas-exchange and measurement of light-dark transient release of CO_2. The lichens *Platismatia glauca, Dermatocarpon miniatum, Dermatocarpon luridum, Peltigera leucophlebia and Roccella phycopsis* were chosen for a detailed investigation of their on-line Δ and gas-exchange characteristics (Table 1). Fresh thalli were collected between May and August 1993 and 1994. Thalli were used immediately or air-dried and stored at − 8 °C. The fresh lichen thalli were kept in a growth room maintained at 17 °C, 75–80% relative humidity (RH) on moist filter paper under a light regime of 30 μmol photon $\cdot m^{-2} \cdot s^{-1}$ with a photoperiod of 12 h. The thalli were sprayed once daily with distilled water. Wherever possible, experimentation was carried out immediately and in all cases was completed within 10 d of collection. Those species which were stored frozen were allowed to reactivate for 48 h under the above conditions before experimentation. No species were kept frozen for more than three weeks.

Carbon isotope analysis

Organic samples. Samples for mass-spectrometric analysis were prepared, combusted and repurified as described previously (Griffiths et al. 1990; Máguas et al. 1993). The resulting samples were analysed using an Isotope Ratio Mass Spectrometer (IRMS) Isospec/VG602 modified by Provac Services, Crewe, UK. More-recently collected specimens were processed automatically, using 0.8-mg samples, in a Europa 20/20 automated IRMS (Europa Scientific Ltd, Crewe, UK).

Data have been calculated as discrimination (Δ) in organic material assuming a $\delta^{13}C$ of source air of − 8‰ versus Pee Dee Belemite (PDB) standard, using Eq. 1 (Farquhar et al. 1989).

$$\Delta = \frac{(\delta_a - \delta_p)}{1 + \delta_p} \qquad \text{(Eq. 1)}$$

This calculation allows a direct comparison with on-line measurements and shows the extent of discrimination in plant material independent of any source CO_2 variations.

On-line samples. The CO_2 samples for mass-spectrometric analysis of $\delta^{13}C$ were collected using an open minicuvette system (Compact Minicuvette System, H. Walz, Effeltrich, Germany), with infrared gas analysers (IRGA) calibrated against CO_2 standards at 0, 345 and 950 μl $\cdot l^{-1}$ (BOC Gases, Brentford, Middx., UK). Samples of lichen thalli of between 2.5 and 4 g fresh weight (area approx. 20 cm^2) were sprayed prior to enclosure within the cuvette and blotted to remove surface water droplets. They were then attached to a perforated, Plexiglas plate perpendicular to the light source. The air passing over the thallus was collected for 15 min via a bypass leaving the Minicuvette system before the analysers. During sample collection the thalli were at saturating irradiances and between 90 and 98% RH. The average water content of the thallus for each collection period of 15 min was obtained by recording the fresh weight of the thallus before and after the sample was collected and expressing the average of these two values as a percentage of the oven dry weight.

The use of a tank-CO_2 supply with a $\delta^{13}C$ significantly different from that of bulk-air CO_2 (currently − 8‰, as opposed to − 40‰ found in most commercial CO_2 sources in the UK) may alter the instantaneous discrimination expressed by the lichen thallus. Compressed air from cylinders was found to provide a constant partial pressure and $\delta^{13}C$ for source CO_2 of around 370 μl $\cdot l^{-1}$ and 10‰ and was used for all experiments. The Minicuvette system was interfaced to a glass sample-preparation line and vacuum system for the collection of CO_2 gas and transfer by cryodistillation using liquid nitrogen. Samples were further re-purified to remove N_2O using the line described above (Griffiths et al. 1990). Reference CO_2 gas samples were taken at regular intervals.

During on-line discrimination, the CO_2 leaving the cuvette is enriched in $^{13}CO_2$ in proportion to the extent of discrimination

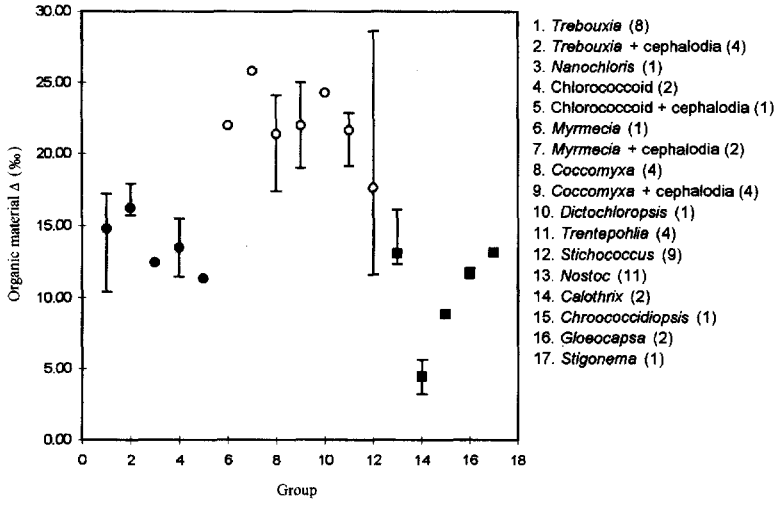

Fig. 1. Relationship between organic-matter Δ and the presence of a pyrenoid in the chloroplast of the primary photobiont in lichens. Organic-matter Δ values are shown for a range of lichens with green algal and cyanobacterial photobionts or a combination of the two (tri-partite lichens). Data are grouped according to the primary photobiont species, and the number of species studied in each group is given in parentheses. Cephalodia contain *Nostoc* with the exception of group 2 which contains *Stigonema. Closed symbols* (●) indicate the presence of a pyrenoid in the photobiont chloroplast; *open symbols* (○) indicate that a pyrenoid is absent; ■ indicates a cyanobacterial photobiont. Mean values are plotted for each group with bars indicating the maximum and minimum values for each group

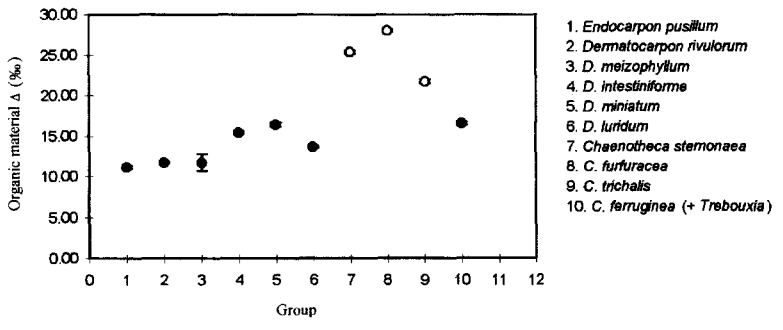

Fig. 2. Organic-matter Δ values for lichens with *Stichococcus* as the primary photobiont. Values are shown for the individual species in group 12 (photobiont = *Stichococcus*) of Fig. 1. Three genera are represented. In addition, Δ is shown for *Chaenotheca ferruginea* which has *Trebouxia* as the photobiont (species no. 10). *Closed symbols* (●) indicate the activity of a CCM implied by the low Δ values (Δ < 16‰). *Open symbols* (○) indicate a C_3-like mechanism implied by the higher Δ values (Δ > 20‰). Mean values for three replicate 8-mg samples are shown with SE

shown by the thallus, which is then derived from the following equation (Evans et al. 1986):

$$\Delta = \frac{\xi\ (\delta_o - \delta_e)}{\delta_o - \xi(\delta_o - \delta_e)}$$

where $\delta_o = \delta^{13}C$ of air leaving the chamber, i.e. $\delta^{13}C$ of analysis gas; $\delta_e = \delta^{13}C$ of air entering the chamber, i.e. $\delta^{13}C$ of reference gas; and $\xi = C_e/(C_e - C_o)$, where C_e = partial pressure of CO_2 entering the cuvette, and C_o = Partial pressure of CO_2 leaving the cuvette.

Gas-exchange

Carbon dioxide response curves. The thallus response to external partial pressures of CO_2 was measured using the Minicuvette system described above with gas supplied by a gas-mixing unit (GMA1; Walz, Effeltrich, Germany) for partial pressures greater than ambient and via a Gas Diluter (ADC, Hoddesdon, UK) for partial pressures less than ambient. Measurements were taken at optimal water contents (Cowan et al. 1992), determined by measuring net assimilation rates (P_{max}) across a range of thallus water contents, and checked by weighing the thallus before and after readings. Saturating irradiances were used. Up to a maximum of three readings at a time could be taken for each thallus sample. Data from between three and five samples of 2.5–4.0 g fresh weight (area approx. 20 cm^2), at partial pressures of 15–1200 $\mu l \cdot l^{-1}$ were used to construct the CO_2-affinity curve for each species. Dark-respiration rates were also measured after P_{max} had been recorded, over the range of CO_2 concentrations.

Measurement of light-dark transients in DIC pools. Small samples of thallus (0.5–1.5 g fresh weight) at optimal water contents were placed in a modified Hansatech leaf-disc gas-phase electrode (LD2; Hansatech, Norwich UK), with the electrode inlet sealed and connected in an open system to an ADC 225 Mk III IRGA (ADC) in differential mode with a reaction time between IRGA and cuvette of 7 s. Ambient air was supplied from a 200-l buffering volume which maintained a constant partial pressure of CO_2, and the water jacket around the oxygen-electrode cuvette was maintained at 15 °C. A chart recorder measured the gas exchange within the "cuvette" and, after a period of dark acclimation when dark respiration rates stabilized, saturating light was supplied using a red light source (LS-2; Hansatech). The resulting gas exchange was recorded and after 10–15 min, when net CO_2 uptake had stabilised, the light was switched off. The chart recorder then traced the return to dark-respiration rates. The experiments were repeated using thalli which had been immersed in 50 mM glycolaldehyde, an inhibitor of photosynthesis, for periods of up to 15 min depending on the hygroscopic properties of the thalli (see Badger et al. 1993).

Chlorophyll analysis

Chlorophyll content was measured for five replicate samples of each species according to Ronen and Galun (1984).

Results

Carbon isotope discrimination (Δ) *in organic material of lichens and bryophytes*

A survey of organic Δ for lichens and bryophytes was used to determine whether categories previously identified from low and high Δ values correlated with the occurrence of a pyrenoid in chloroplasts and the activity of a CCM. *The relation between pyrenoid and CCM in lichens.* Figure 1, containing analyses of 170 samples from 58 species

Table 1. Organic material Δ for selected photosymbiodemes. Data represent at least three replicate 8-mg samples and show mean values ± SE. All samples were from the Herbarium of the Royal Botanic Gardens Edinburgh

Species	Authority	Comments	Thallus portion	Primary photobiont	Organic Δ
Sticta canariensis	Bory ex Delise (1823)	Green algal morphology	All	*Myrmecia*	26.51 ± 0.02
Sticta duforii	Delise (1823)	Cyanobacterial morphology	All	*Nostoc*	16.95 ± 0.12
Sticta duforii		Composite thallus	Green algal leaflet	*Myrmecia* on *Nostoc*	16.67 ± 0.34
Solorina spongiosa	(Ach.) Anzi (1862)	Green alga restricted to apothecial collar on cephalodial cushion	All	*Nostoc*	11.83 ± 0.08
			Apothecial collar	*Coccomyxa*	16.20 ± 1.18
Solorina crocea	(L.) Ach (1810)	Thallus continuously layered with *Coccomyxa* above *Nostoc*	All	*Coccomyxa* + *Nostoc*	22.61 ± 0.08
			Upper layer	*Coccomyxa*	22.23 ± 0.14
			Lower layer	*Nostoc*	22.72 ± 0.15
Lobaria amplissima	(Scop.) Forss. (1883)	Thallus with large shrubby external cephalodia	All	*Coccomyxa* + *Nostoc*	25.60 ± 0.03
			External cephalodia	*Nostoc*	22.19 ± 0.03
Dendriscocaulon umhausense	(Auersw.) Degel. (1942)	Free-living cephalodia of *Lobaria amplissima*	All	*Nostoc*	17.21 ± 0.32

of lichen, relates thallus organic Δ to the taxonomy of the algal partner, the presence of a secondary photobiont in cephalodia and the presence of a pyrenoid in the chloroplast. The species in groups 1 and 2 have *Trebouxia* spp. as their primary photobiont but include foliose, fruticose and crustose life-forms. All have organic Δ values ranging from 10.11 ± 0.30 to 17.60 ± 0.15‰, which is mid-way between the range normally associated with C_4 and C_3 terrestrial vascular plants. Species in group 2 have cephalodia with the cyanobacterium *Stigonema* as the secondary photobiont. There is no significant difference between the organic Δ values exhibited by species with or without cephalodia. In group 3, *Nanochloris*, like *Trebouxia*, has a pyrenoid within the chloroplast, and Δ of 12.31 ± 1.41‰. Species in groups 4 and 5 have a chlorococcoid photobiont, probably *Chlorella* or *Pseudochlorella*, with pyrenoid (Tschermak-Woess 1982).

Groups 6–11 represent species which lack a pyrenoid in the chloroplast. Discrimination is high in species with *Coccomyxa*, *Dictochloropsis* and *Myrmecia* as primary photobionts and *Nostoc* in cephalodia, and also in lichens with *Coccomyxa* or *Trentepohlia*, where cephalodia are absent. Species in groups 13–17 are cyanobacterial lichens, known to have a CCM (Badger et al. 1993; Máguas et al. 1993). They exhibit Δ values ranging fom 3.41 ± 0.42‰ for the frequently immersed *Lichina pygmaea*, to 16.11 ± 0.94‰ for *Nephroma laevigatum*.

The Δ values obtained for group 12 are equivocal, ranging between 11.02 ± 0.19 for *Endocarpon pusillum* and 28.41 ± 0.10‰ for *Chaenotheca furfuracea*. Figure 2 shows the results for each individual lichen in this group containing *Stichococcus*. The species fall clearly into two groups with members of the genera *Endocarpon* and *Dermatocarpon* exhibiting low, C_4-like Δ values whereas members of the genera *Chaenotheca* have C_3-like Δ values. *Endocarpon pusillum* is known to have *Stichococcus diplosphaera* (Bialosuknia) Chodat (Chodat 1913, quoted in Ahmadjian and Heikilla 1970) (with pyrenoid) as the photobiont. At present the taxonomy of the photobiont of species in the genus *Dermatocarpon* is uncertain although it is likely to be *Stichococcus* (Ahmadjian 1967; Ahmadjian and Heikilla 1970; Tschermak-Woess 1982). There is also some confusion as to whether *Stichococcus* has a pyrenoid, with reports that a pyrenoid is lacking (Ahmadjian 1967; Tschermak-Woess 1982), and reports that pyrenoids and pyrenoglobuli may be present in some species of *Stichococcus* (Ahmadjian 1993).

We have confirmed that a pyrenoid is present in the chloroplast of *Dermatocarpon luridum* by confocal fluorescence microscopy (data not shown). This provides conclusive evidence that the activity of a CCM and low, C_4-like organic Δ is associated with the presence of a pyrenoid. We suggest that the photobiont of *Chaenotheca* is in fact a species which does not possess a pyrenoid, and this has been confirmed by preliminary microscopic investigation. It should be noted that structurally the genus *Chaenotheca* is very different from the genera *Endocarpon* and *Dermatocarpon*, *Chaenotheca* being a crustose species and the latter two being foliose. However, structural differences alone are unlikely to account for the observed variation in organic Δ, since *Chaenotheca ferruginea*, with *Trebouxia* as the photobiont, has a much lower Δ value than the other species in the genus (Δ = 15.84 ± 0.12‰, Fig. 2).

In order to examine the relationship between photobiont, pyrenoid and Δ, the investigation was extended to include Δ of lichens in which the mycobiont associates with more than one alga within the same thallus (photosymbiodemes) see (James and Henssen 1976; Purvis et al. 1992) (Table 1). In *Sticta canariensis* the mycobiont may associate with a chlorococcoid alga (probably *Myrmecia*) or *Nostoc* (as *S. duforii*). The organic Δ values reflect the activity of the cyanobacterial CCM in *S. duforii* (Δ = 16.95 ± 0.12‰). In *S. canariensis*, organic Δ is more C_3-like (26.50 ± 0.02‰) implying the absence of a CCM. However, for a composite thallus of *S. duforii*, upon which grow leaflets containing only *Myrmecia*, the organic Δ reflected carbon from the primary photobiont of the main thallus, *Nostoc* (Δ = 16.67 ± 0.34‰; Table 1). There is a similar gradation of Δ in the lichens *Solorina spongiosa* and *S. crocea*, the former having *Coccomyxa* restricted to a collar on a cephalodial cushion of *Nostoc*, the latter having a continuously layered thallus with *Coccomyxa* above *Nostoc* (Table 1).

Furthermore, organic Δ of the large, external, cephalodia of *Lobaria amplissima* containing *Nostoc*, *when removed from the thallus, reflected that of the primary photobiont Myrmecia* rather than the cephalodial *Nostoc* (Δ for whole thallus = 25.6 ± 0.03‰, Δ for external cephalodia = 22.19 ± 0.03‰ (Table 1)). However, for free-living

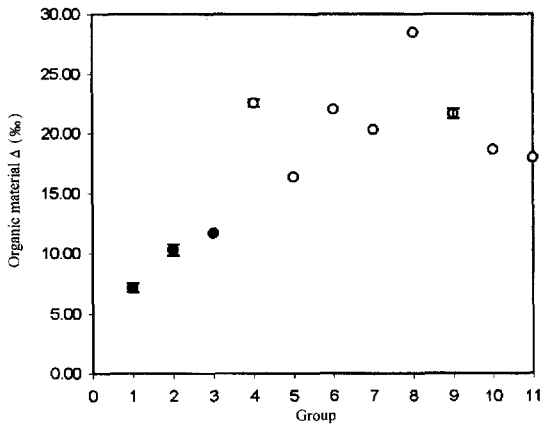

Fig. 3. Relationship between organic-matter Δ values for selected Bryophytes and the presence of a pyrenoid. Data are shown for a range of hornworts (species 1–3), liverworts (species 4–7) and mosses (species 8–11). Data points represent mean values of at least four replicate samples from different plants ± SE. *Closed symbols* (●) represent species with a pyrenoid in the chloroplast. *Open symbols* (○) represent species without a pyrenoid in the chloroplast

Table 2. Characteristics of species studied in detail

Species	Authority	Photobiont genus	Pyrenoid	Habitat
Platismatia glauca	(L.) Culb. & C. Culb	Trebouxia[1,2,3]	Present	Deciduous woodland
Peltigera leucophlebia	(Nyl.) Gyelnik	Coccomyxa + Nostoc[1,2,3]	Absent	Grassland
Dermatocarpon miniatum	(L.) Mann	Stichococcus?[1,4]	Present	Limestone outcrop
Dermatocarpon luridum	(With.) Laundon	Stichococcus?[1]	Present	Upland stream
Roccella phycopsis	Ach.	Trentepohlia[1,2,3]	Absent	Coastal rocks

[1] Tschermak-Woess 1982
[2] James and Henssen 1976
[3] Ahmadjian 1967
[4] Purvis et al. 1992

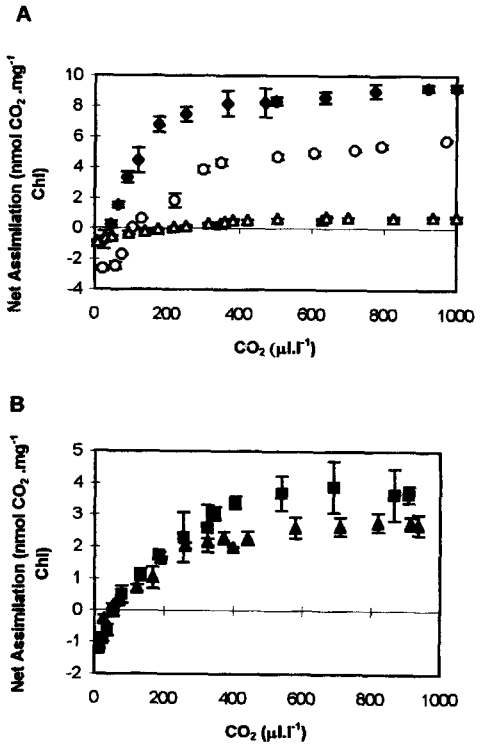

Fig. 4A, B. Carbon dioxide response curves for representatives of different photobiont associations. Net assimilation rates at varying external (cuvette) concentrations of CO_2 for the five species of lichen studied. ♦, *Platismatia glauca*; ○, *Peltigera leucophlebia*; △, *Roccella phycopsis*; ■, *Dermatocarpon luridum*; ▲, *Dermatocarpon miniatum*. Data represent mean values of at least four readings at each CO_2 concentration ($n = 3$ for *R. phycodes*) ± SE

cephalodia of *Lobaria amplissima* (*Dendriscocaulon umhausense*), thallus organic Δ was found to be more C_4-like (Δ = 17.21‰). This indicates that, in the free-living state, when dependent upon photosynthesis by the cyanobacterial photobiont alone, the organic Δ of cephalodia reflects the activity of the cyanobacterial CCM.

When the survey was extended to include bryophytes, similar relationships were found (Fig. 3). It was evident that low Δ of the thallus organic material correlated with the presence of a pyrenoid in the chloroplast, (see *Anthoceros* and *Phaeoceros*, where Δ = 9.13 ± 0.11‰ and 10.40 ± 0.75‰, respectively). *Blasia pusilla*, a member of the Hepaticae which also has small, ventral *Nostoc*-filled cavities but no pyrenoid in the chloroplast had an organic-matter Δ value of 23.34 ± 0.55‰ (Fig. 2), comparable to that of the majority of C_3-like Hepaticae (e.g. *Pellia* Δ = 23.70 ± 0.31‰). Thus, as with the cephalodia of lichens, the *Nostoc* contained within the thallus of these bryophytes does not contribute significantly to thallus carbon accumulation.

Photosynthetic characteristics and CCM activity in selected photobiont associations

A small number of lichen associations representative of these groups were studied in detail with regard to photosynthetic characteristics (for taxonomy and habitat preference see Table 2).

Exchange of CO_2 and carbon affinity. Uptake responses of CO_2 for each of the species confirmed that CO_2 affinity was related to organic Δ values (Fig. 4), even when net assimilation rates were expressed per unit chlorophyll, per unit dry weight (g DW) and per unit area (Table 3). The Trebouxioid lichens, with low Δ, had a higher affinity for CO_2 and a lower thallus and photobiont compensation point (Γ) than the other species ($K_{0.5} = 195\ \mu l \cdot l^{-1}$, $\Gamma = 44\ \mu l \cdot l^{-1}$; Fig. 4A). Lichens with *Stichococcus* as photobiont had CO_2 affinities similar to those with *Trebouxia* as the photobiont ($K_{0.5} = 200$–$215\ \mu l \cdot l^{-1}$, $\Gamma = 45$–$48\ \mu l \cdot l^{-1}$ (Fig. 4B)). Our data confirm the absence of a CCM in *Coccomyxa* and indicates that the CCM is also absent in lichens with *Trentepohlia* as the photobiont ($K_{0.5} = 400\ \mu l \cdot l^{-1}$, $\Gamma = 120\ \mu l \cdot l^{-1}$), together with higher Δ characteristics described above (Fig. 1). Despite the activity of a CCM, the net assimilation rates of the two *Dermatocarpon* species are lower than those of *Peltigera leucophlebia* which possesses *Nostoc* in cephalodia (Table 3).

Direct measurement of DIC pools during light-dark transients. The measurement of light-to-dark transient uptake and release of CO_2 (Badger et al. 1993) has been used to identify the presence of a DIC pool in a number of cyanobacterial lichens and lichens with *Trebouxia* as the photobiont. (Badger et al. 1993; Lange et al. 1994; Palmqvist et al. 1994a). We have now extended this analysis for lichens with *Trebouxia*, *Coccomyxa* + cephalodia, *Stichococcus* and *Trentepohlia*, so as to compare the effect of cephalodia and pyrenoid in the chloroplast (Fig. 5). The control treatments show an initial dark phase as each thallus attained a steady rate of dark respiration. Upon

Table 3. Gas-exchange characteristics of species used for detailed study. Net assimilation rates represent maximum values obtained at optimal thallus water content, saturating light, ambient partial pressure of CO_2 and 15 °C. Data represent at least four replicates and show the SE. $K_{0.5}$ values (external concentration of CO_2 at which half-maximal rates of CO_2 assimilation are reached) and CO_2 compensation points are measured from the CO_2 response curves (Fig. 4)

Species	Net assimilation nmol $CO_2 \cdot (g\,DW)^{-1} \cdot s^{-1}$	nmol $CO_2 \cdot (mg\,Chl)^{-1} \cdot s^{-1}$	$\mu mol\,CO_2 \cdot m^{-2} \cdot s^{-1}$	Dark respiration nmol $CO_2 \cdot (g\,DW)^{-1} \cdot s^{-1}$	$K_{0.5}$ $(\mu l \cdot l^{-1})$	Compensation point $(\mu l \cdot l^{-1})$
Platismatia glauca	7.43 ± 0.43	6.6 ± 0.01	1.76 ± 0.06	0.99 ± 0.02	195	44
Peltigera leucophlebia	3.65 ± 0.29	3.56 ± 0.08	0.95 ± 0.09	0.56 ± 0.31	388	85
Dermatocarpon miniatum	2.78 ± 0.72	2.46 ± 0.12	0.58 ± 0.01	0.67 ± 0.08	215	45
Dermatocarpon luridum	2.99 ± 0.24	2.31 ± 0.09	0.67 ± 0.10	0.91 ± 0.12	200	48
Roccella phycopsis	1.02 ± 0.89	0.91 ± 0.10	fruticose	0.95 ± 0.10	400	120

illumination net photosynthesis increased to a maximum value over 10 min. *Platismatia glauca* showed an initial burst of CO_2 uptake (seen as a "spike" in Fig. 5a) followed by a gradual increase to the maximum value. After the transition to darkness a small pool of CO_2 was released and after 1–3 min the steady rate of dark respiration was resumed (Fig. 5a). *Peltigera leucophlebia* (Fig. 5c) consistently showed a second slow peak in CO_2-uptake rate before maximum photosynthesis was attained and a small release of CO_2 on the transition to darkness. Species of *Dermatocarpon* failed to show any initial uptake peak or post-illumination release of CO_2 (Fig. 5g, e). *Roccella phycopsis* also did not show any uptake peak or post illumination release of CO_2 (Fig. 5i).

Figure 5 also shows the response of all species after immersion in glycolaldehyde, an inhibitor of photosynthesis which enables any light-driven accumulation of DIC to be identified. Thus the extent of any uptake and release of DIC normally masked by CO_2 exchange can be revealed (Fig. 5b, d, f, h, j). Table 4 shows the integrated pool size for each species. *Platismatia glauca*, *Dermatocarpon miniatum* and *Dermatocarpon luridum* accumulated pools of between 24 and 51 $nmol \cdot mg^{-1}$ Chl as measured by the post-illumination release of DIC in thalli treated with glycolaldehyde. *Peltigera leucophlebia* and *Roccella phycopsis* accumulated much smaller pools (8.0 ± 2.4 and 6.0 ± 2.1 $nmol \cdot mg^{-1}$ Chl, respectively).

Activity of the CCM and on-line Δ characteristics. In order to integrate the CCM activity across the working range of thallus water contents and determine the extent that Δ varies during photosynthesis, gas-exchange and on-line Δ were compared for each of the selected lichen associations (Fig. 6). The results of these studies were consistent with previous studies using *Trebouxia* and *Coccomyxa* as the primary photobiont (Palmqvist et al. 1994b; Máguas et al. 1995). Maximum rates of net photosynthesis were reached at thallus water contents of 250–400% for both groups, without any significant reduction at higher water contents. In absolute terms the maximum net rates for the *Trebouxioid* lichens (Fig. 6a) were greater than those with *Coccomyxa* (Fig. 6b) when measured per unit DW and per unit chlorophyll (see also Table 3). There was a significant difference between on-line Δ for the two photobionts at optimal water contents with the Trebouxioid lichen having a Δ value of 13.28 ± 1.53‰ compared with 23.21 ± 0.61‰ for the lichen with *Coccomyxa*. This is consistent with a CCM operating in lichens with *Trebouxia* as the photobiont but not in lichens with *Coccomyxa* as the photobiont.

At water contents greater than 300% for *Platismatia glauca* and 350% for *Peltigera leucophlebia* there was a small but significant decrease in Δ values, from a maximum of 13.28‰ to 11.13‰ for *Platismatia glauca* (*Trebouxia*) and from 23.21‰ to 19.83‰ for *Peltigera leucophlebia* (*Coccomyxa*). Similarly, at water contents less than 200% there was a decrease in Δ for both groups again in the order of 3‰, but this time accompanied by a decrease in net assimilation rates of approximately 2 nmol $CO_2 \cdot mg^{-1}$ Chl. The magnitude of the variation in Δ caused by fluctuations in thallus water content is not sufficient to account for groups with different organic

material Δ (Fig. 1) arising from diffusion limitation alone (Máguas et al. 1995) and there was close agreement between organic and on-line Δ for these species.

Both species of *Dermatocarpon* (*Stichococcus*) showed similar responses (Fig. 6c, d), with optimal thallus water contents between 250% and 300% and maximal net rates of assimilation 2.78 ± 0.72 (*D. miniatum*) and 2.99 ± 0.24 (*D. luridum*) nmol $CO_2 \cdot g^{-1}$ DW $\cdot s^{-1}$. On-line Δ values of 24.13 ± 1.04‰ (*D. miniatum*) and 25.43 ± 1.76‰ (*D. luridum*) corresponded with organic-matter Δ of 16.75 ± 0.32 and 11.61 ± 0.06‰, respectively (Table 3, Fig. 6c, d).

The results for *Roccella phycopsis* (photobiont *Trentepohlia*: Fig. 6e) extended over a more-limited range of water contents (130%–180%) due to the low rates of net assimilation found for this species. Nevertheless, it is apparent that Δ is in the upper part of the range

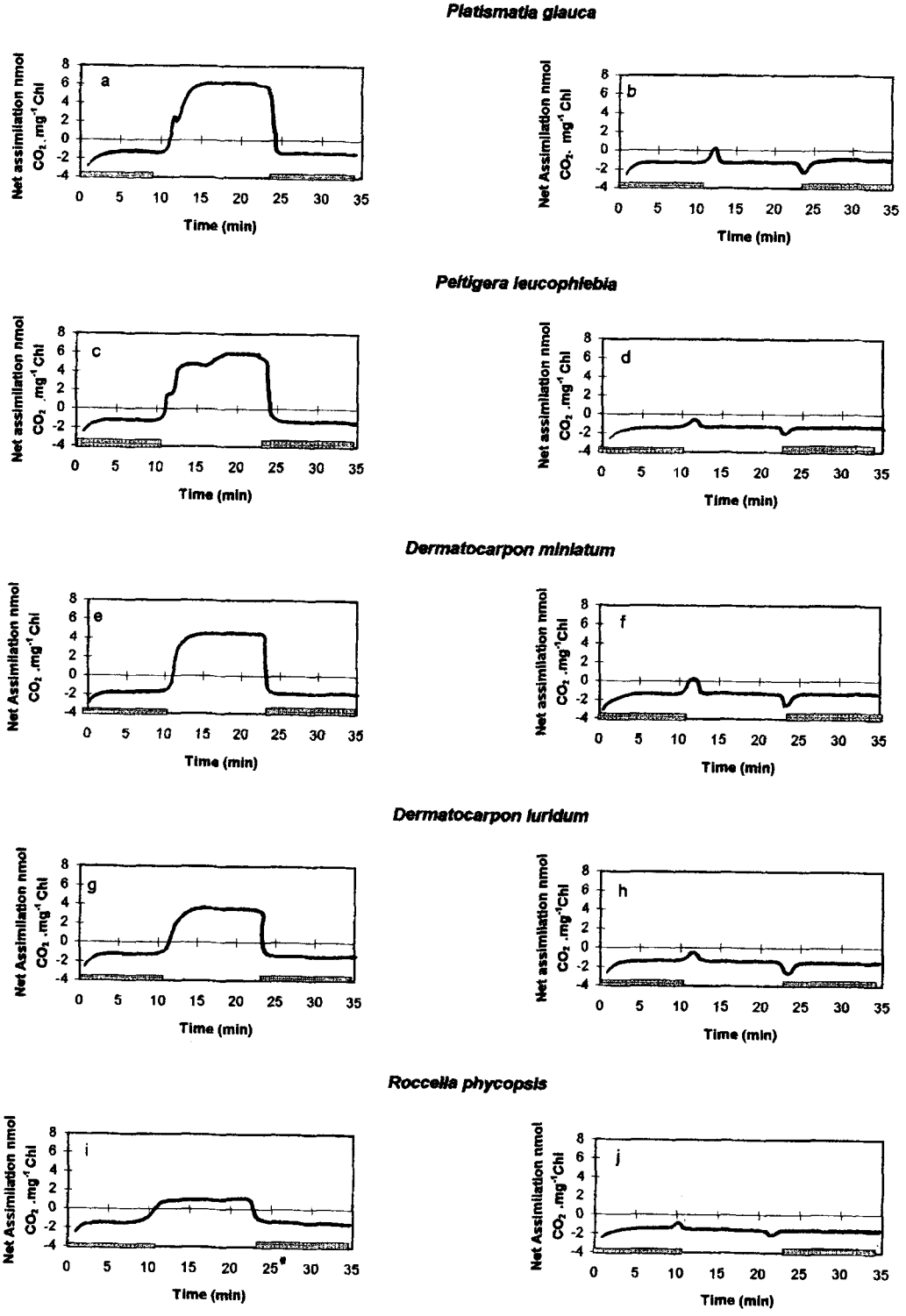

Fig. 5a–j. Accumulation and release of internal pools of CO_2 during light-dark transients. Net assimilation rates during a period of darkness (*shaded horizontal bar along time axis*) are followed by illumination (*no horizontal bar*) and finally another period of darkness (*shaded bar*). Typical traces are shown for each species. Five experimental recordings were made for each species after immersing the thalli in distilled water for 20 min (**a, c, e, g, i**), followed by five further recordings after immersing the same thalli in 50 mM glycolaldehyde **b, d, f, h, j**

Table 4. Magnitude of internal pool of CO_2 accumulated and released during light-dark transients. The magnitude of the pool accumulated during illumination was calculated by integrating the area between the initial uptake peak and the photosynthetic response trace. An initial uptake peak was present for *Platismatia glauca* only in the absence of glycolaldehyde treatment. After treatment with glycolaldehyde (see text) initial uptake peaks were present for all species. A similar calculation of the area of curve between the final CO_2 release and the photosynthetic response trace which would be predicted had dark respiration resumed immediately was used to measure the post-illumination CO_2 release of the internal pool of CO_2. No species showed post-illumination release of CO_2 unless immersed in glycolaldehyde. Distilled water was used for immersion in the control experiment compared with immersion in 50 mM glycolaldehyde. Data represent mean values ± SE from five replicate experiments

Species	Initial CO_2 uptake ($nmol \cdot mg\,Chl^{-1}$)		Post-illumination CO_2 release ($nmol \cdot mg\,Chl^{-1}$)	
	Control	Glycolaldehyde	Control	Glycolaldehyde
Platismatia glauca	20.0 ± 1.53	31.5 ± 2.10	–	51 ± 2.8
Peltigera leucophlebia	–	12.3 ± 1.22	–	8 ± 2.4
Dermatocarpon miniatum	–	21 ± 0.97	–	24 ± 1.9
Dermatocarpon luridum	–	28 ± 3.2	–	34 ± 2.5
Roccella phycopsis	–	7.5 ± 1.5	–	6 ± 2.1

characteristic of C_3 higher plants. There is, however a discrepancy between the maximum on-line values and the organic-material Δ values. On-line Δ of 33.15 ± 0.64‰ corresponded with organic-matter Δ of 20.81 ± 0.21‰ for *Trentepohlia*. Further work is needed on the effect of refixation of respiratory CO_2 on on-line Δ since the lichens with *Stichococcus* and *Trentepohlia* had high rates of dark (predominantly mycobiont) respiration relative to their maximum net rates of photosynthesis (Table 3) see also Rice and Giles (1994), and Máguas et al. (1995).

Discussion

We may now confirm that the activity of a CCM is related to the categories of lichens identifiable from the Δ values of thallus organic material. This develops the suggestions in previous work that two groups, with green and blue-green photobionts were distinct (Lange and Ziegler 1986; Lange 1988; Lange et al. 1988) and subsequently that three groups, including blue-green, green and tri-partite lichens (Máguas et al. 1993, 1995), were identifiable. It is apparent from Fig. 1 that cyanobacterial lichens and those lichens in which the photobiont has a pyrenoid in the chloroplast [*Trebouxia*, *Stichococcus* (some species), *Nanochloris*, *Chlorella* and *Pseudochlorella*] have low, more C_4-like Δ values and strong evidence for a DIC pump from gas-exchange measurements. In contrast, those species where the photobiont is a green alga without a pyrenoid in the chloroplast [*Myrmecia*, *Stichococcus* (some species) *Coccomyxa*, *Dictochloropsis*] have a characteristically C_3-like Δ. This latter group includes many of the tri-partite lichens previously investigated, but the presence of cephalodia containing *Nostoc* in many lichens which have *Trebouxia* as a photobiont does not per se result in a C_3-like Δ. Additionally, this implies that CO_2 fixation by the cyanobacterium is negligible when compared with the total organic-matter carbon content of the thallus. This was supported by data relating to the organic-matter Δ of photosymbiodemes in the genera *Lobaria*, *Sticta* and *Solorina* (Table 1).

The hypothesis that the pyrenoid is involved in the activity of the DIC pump is supported by data from species of bryophytes. Here the Anthocerotae, possessing a pyrenoid within the chloroplast, exhibited more C_4-like Δ values which were lower than those of many of the cyanobacterial lichens. These were markedly different from the C_3-like Δ values obtained for other bryophytes, including *Blasia pusilla*.

The conclusion that species with a pyrenoid possess an active CCM is supported by gas-exchange measurements and the measurement of light-dark transient releases of the DIC pool. Although all species showed a degree of accumulation and release of DIC during the light-dark transient measurements, the magnitude of the release in those species with high organic Δ values was less than in the species with low Δ values, and was more in the range to be expected by light-driven alkalinisation of the stroma (Badger et al. 1993). Additionally, species of lichen with a pyrenoid in the algal chloroplast had generally higher net assimilation rates than those without, particularly at low external concentrations of CO_2, the exception being when the association included *Nostoc* in cephalodia. Furthermore CO_2 compensation points were lower, in the region of 40–50 $\mu l \cdot l^{-1}$, compared with 85–120 $\mu l \cdot l^{-1}$ for associations lacking a pyrenoid in the algal chloroplast. This supports the findings of Máguas et al. (1993, 1995) who used tri-partite lichens with *Myrmecia* and *Coccomyxa* as primary photobionts and Palmqvist et al. (1994a, c) using lichens with *Trebouxia*, *Coccomyxa* and *Nostoc* as primary photobionts.

We have also been able to quantify the contribution of diffusion limitation to variations in organic Δ using instantaneous, on-line Δ. Diffusion limitation caused depressions in Δ of around 2–3‰ in water-saturated thalli. However any variation in DIC pump activity with high thallus water content may also appear as a reduction in Δ and would be confirmed by an increase in the size of the DIC pool of CO_2 that is released (Lange et al. 1994; and data not shown). It is therefore possible that reductions in Δ at high water content are not caused solely by the effects of diffusion limitation. Work is currently underway to relate depression of Δ at high thallus water contents to the size of the DIC pool, using a combination of on-line Δ and measurement of light-dark transients. The effects of respiratory CO_2 on the on-line

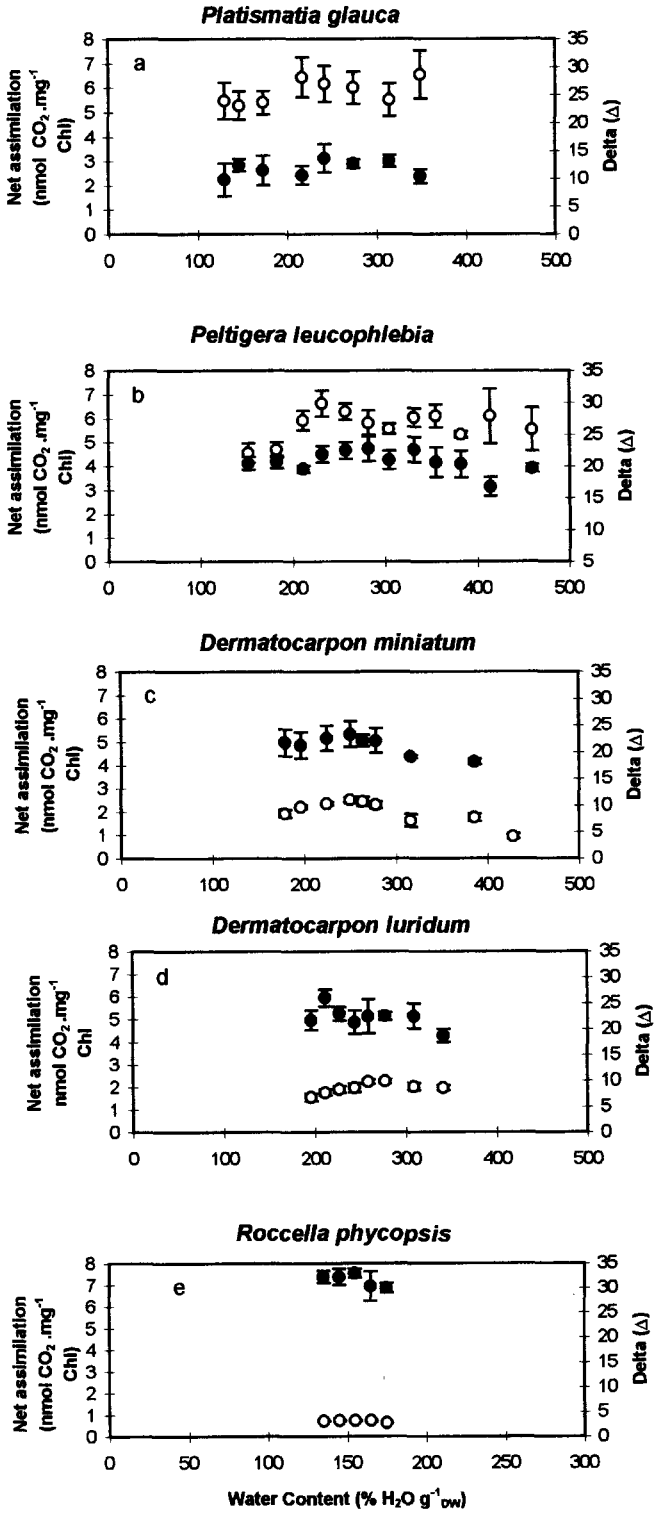

Fig. 6a–e. On-line Δ values (●) and net CO_2 assimilation rates (○) over a range of thallus water contents for lichens representing contrasting photobiont associations. Data points represent a running mean taken with $n = 5$ for each point and showing SE

signal should also be noted since in the three species which show a high rate of repiration (predominantly mycobiont respiration) there is a discrepancy between organic and on-line Δ.

Finally, a number of authors have linked the presence of nitrogen-fixing cyanobacteria with the absence of the CCM. Although it has been shown that under conditions where nitrogen supply is limiting a green algal CCM remains active even under high partial pressures of CO_2 (Beardall et al. 1982) the data presented here do not support the correlation between the absence of a CCM and the presence of a nitrogen supply from fixation to compensate for any losses due to photorespiration. It is apparent that the "C_3-like" lichens include examples of photobionts which do not associate with cephalodia (*Trentepohlia*) as well as photobiont species which form tri-partite associations and also occur as the sole photobiont, (e.g. *Coccomyxa* in *Baeomyces placyphyllos*). Conversely, cephalodia occur in a number of species in which the organic Δ value indicates that a CCM is operating (e.g. *Placopsis gelida*, *Stereoacaulon vesuvianum* and *S. dactylophyllum*). Further work on the photorespiratory characteristics of species with a CCM and cephalodia, and those without the CCM and lacking cephalodia, is needed. We may then be able to reconcile the presence of cephalodia and the CCM to the suggested high nitrogen requirement on the part of the mycobiont of lichens in the genus *Stereocaulon* (Crittenden 1991).

Conclusion

The presence of low organic Δ values in lichens and bryophytes correlates well with the presence of a pyrenoid in the algal chloroplast. Recent research into the ultrastructure and biochemistry of the pyrenoid has provided further insight into the mechanism whereby DIC may be supplied to the Rubisco located within the pyrenoid (Badger and Price 1992; Pronina and Semenenko 1992). Our data confirm that the pyrenoid is likely to play a central role in the concentrating and fixation of CO_2 in chloroplasts. The more C_4-like characteristics of lichen photobionts which have been identified through organic Δ are confirmed in terms of carboxylation efficiency, the $K_{0.5}$ of CO_2 exchange and by the magnitude of the DIC pool accumulated. Thus, under field conditions it is unlikely that the effects of diffusion limitation on the thallus is the sole cause of the lower organic Δ expressed by the lichens with *Trebouxia*, *Nanochloris*, *Chlorella*, *Pseudochlorella* and *Stichococcus* or cyanobacteria as the primary photobiont.

The screening of organic-matter Δ for a variety of lichen associations can identify the presence of a CCM. However, interactions between the extent of respiratory refixation still need to be elucidated with reference to the long-term regulation of the CCM by environmental factors and the effect on the instantaneous on-line signal.

We are currently investigating the suitability of Anthocerotae of the genera *Anthoceros* and *Phaeoceros* as species in which the activity of the CCM associated with a pyrenoid may be examined in more detail using gas-exchange and on-line Δ. These species also possess other features of interest such as stomata on the sporophyte and *Nostoc* within cavities in the thallus but they do not feature the complicating interrelationship with a symbiotic fungus that occurs in the lichens. Bryophytes may

thus prove suitable organisms for which the detailed operation of the CCM may be investigated using the Δ and gas-exchange techniques described above.

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