Growth, nitrogen uptake, and resource allocation in the two tripartite lichens *Nephroma arcticum* and *Peltigera aphthosa* during nitrogen stress

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Summary

• Lichen responses towards nitrogen stress, both increased exposure and deprivation of N, were investigated by measuring N uptake, growth, ergosterol, chitin and Chl*a* in two tripartite nitrogen-fixing species, *Nephroma arctium* and *Peltigera aphthosa*.
• The lichens were irrigated with different N forms, enriched in ^{15}N to assess N uptake, during 3 months in the field, with a total N dosage of 500 mg m^{-2}. Nitrogen deprivation was induced by removing the nitrogen-fixing cephalodia.
• The lichens took up 11–134 mg N m^{-2} of the added N, corresponding to 1–4% of their total thallus N. Uptake was 4 times higher for NH_4^+ than for NO_3^-, and the highest ^{15}N concentrations were found in newly synthesized tissue. Both forms of N stress affected thallus expansion rates in both species.
• It is concluded that the two lichens were able to maintain a balanced tissue N concentration despite large variations in N supply, and that assimilated N might be transported to growing apices. Alternatively, N assimilation from external sources might be greater in the margins than in the mature thallus. Thallus expansion was sensitive to N stress, apparently being tightly coupled to N assimilation.

Key words: ^{15}N, lichen growth, resource allocation, nitrogen uptake, ergosterol, chlorophyll, chitin, nitrogen stress.

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Introduction

Lichens are symbiotic organisms where the heterotrophic partner, a fungus (mycobiont) derives carbon, and in some cases nitrogen, from algal and/or cyanobacterial photobionts. Symbiotic organisms, such as lichens, are complex since their maintenance and function depends on co-ordinated growth between the partners (Brown, 1985; Honegger, 1991). Because drastic environmental changes can affect the lichen partners differently, a functional response towards such changes possibly requires a tight regulation of resource investment between the bionts to maintain balanced growth (cf. Sundberg *et al.*, 2001). One stress factor that has received increased attention during recent years is the increased anthropogenic N loads. Indeed, in the path of increasing N deposition, several lichen species have declined in numbers or simply disappeared from known habitats, leaving a lichen flora of nitrophilous species (Hallingbäck, 1991).

The perseverance of certain lichen species and the disappearance of others indicate interspecific differences in physiological responses towards N availability. Lichens may for instance differ with respect to N requirements since green algal lichens generally have low thallus N concentrations while cyanobacterial (*Nostoc*) lichens with their N_2-fixation ability have higher N availability (Rai, 1988; Palmqvist *et al.*, 1998). It has also been shown that different lichens allocate their N resources differently, where lichens associated with green algae invest a larger fraction of their N into photosynthetic tissue, cyanobacterial lichens invest a larger fraction of their N into fungal tissue (Palmqvist *et al.*, 1998). Such differences in N requirements and its allocation may affect the tolerance of lichen species towards increased N deposition.

It appears that N_2-fixing species are particularly affected by N deposition (Rai, 1988; Hallingbäck, 1991). For instance, addition of ammonium (NH_4^+) decreased the rate of cephalodial N_2 fixation in *Peltigera aphthosa* (Rai *et al.*, 1981; Hällbom &

Table 1 Abbreviations for the nitrogen manipulations

Nitrogen fertilization/deprivation	Treatment abbreviation
None	C
NH_4Cl	NH_4^-
KNO_3	NO_3^-
$NH_4NO_3 + KH_2PO_4$	NH_4NO_3 + P
NH_4NO_3	NH_4NO_3
Cephalodial removal	$-N_{fix}$
Cephalodial removal with addition of $NH_4NO_3 + KH_2PO_4$	$-N_{fix}$ NH_4NO_3 + P

Bergman, 1983), indeed also resulting in a decreased N export into adjacent hyphae (Rai *et al.*, 1981). On the other hand, N in the form of nitrate (NO_3^-) did not lower the N_2 fixation rate in the same lichen species (Hällbom & Bergman, 1983). This differential response with respect to N source suggests that the two forms affect N_2-fixation in *Nostoc* in separate ways. Moreover, it has been shown that addition of phosphorus decreases the inhibition of N_2-fixation in cephalodia (Crittenden *et al.*, 1994) implying that this compound, as well as other minerals, can alter the effect of deposited N (Honegger, 1991).

In addition to inhibiting N_2-fixation, N provided from external sources might be assimilated with different efficiency of the lichen partners, or be allocated differently within the thallus, responses that may result in resource imbalances between the lichen bionts. The ability to regulate N assimilation, when environmental supply, is altered is then another factor determining the actual effect of deposited N on lichens. In spite of this, studies on N assimilation by lichens are scarce. Among these, most have followed short-term responses (Rai *et al.*, 1981), while only a few have investigated longer-term assimilation in the field (Hyvärinen & Crittenden, 1998).

The aim of this study was to assess the assimilation and allocation of externally added N for the N_2-fixing tripartite lichen species *Peltigera aphthosa* and *Nephroma arcticum* during 3 months growth in the field. Additionally, we wanted to investigate the species response to the stress of N deficiency, achieved by removing their N_2 fixing cephalodia (Sundberg *et al.*, 2001). Alterations in resource allocation patterns between the bionts were investigated by quantifying chlorophyll *a* as a marker for the photobiont (Raven, 1992), chitin for fungal biomass (Ekblad & Näsholm, 1996), and ergosterol for lichen respiratory load (Sundberg *et al.*, 1999). The lichen's growth patterns with respect to relative weight and area gain were followed to assess their overall response to the two forms of N stress.

Materials and Methods

Lichen material

Nephroma arcticum (L.) Torss. was collected at Kulbäcksliden and *Peltigera aphthosa* (L.) Willd. at Svartberget, outside Vindeln in the county of Västerbotten, Sweden in early June 1999. Both species are tripartite lichens having green algal *Coccomyxa* sp. as the primary photobiont and an N_2 fixing *Nostoc* sp. in cephalodia. For brevity, each lichen will be referred to as 'a species' in the text.

Transplantation

After collection, all thalli were lightly sprayed with water and rinsed from debris. Seventy healthy-looking thalli of each species were chosen for the experiment. The initial d. wt of each was determined to the nearest 0.1 mg after drying in darkness in a climate-controlled room (15°C, 30–40% rh) until no further change in weight could be detected (*c.* 48 h). Each thallus was thereafter sprayed with water to determine its area when fully expanded and hydrated (Palmqvist & Sundberg, 2001). Dry weights varied between 0.1 and 1 g, area between 15 and 80 cm^2, and thallus specific weights (TSW) between 70 and 180 g m^{-2}, among the individuals of both species.

Of the 70 transplants of each species 10 were randomly assigned to one of the seven treatments (Table 1). All thalli assigned for transplantation were mounted on a nylon net frame fixed with four flexible aluminium rods as in previous studies (Palmqvist & Sundberg, 2000; Sundberg *et al.*, 2001). Thalli assigned to each treatment were mounted on one frame each (0.25 m^2), with the two species adjacent to each other. In the field, the seven frames were placed as close together as possible, avoiding cross-contamination of the different N additions, but in similar microclimatic conditions, on the moss covered forest floor, and exposed to the sky. The transplantation site was the same mixed and relatively open Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) forest stand at Ulterviken (63°48′ N, 20°27′ E) as in previous studies (Palmqvist & Sundberg, 2000; Sundberg *et al.*, 2001). The experiment started on 23 June 1999, and ended on 29 September 1999. After harvest, d. wt and area of each thallus were determined as before transplantation (see above).

Nitrogen manipulation

Nitrogen stress, expressed as N deficiency, was obtained by removing all visible cephalodia before transplantation, and thereafter throughout the transplantation period, using a sharp razor blade. The cephalodia covered 2–5% of the surface

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area in *P. aphthosa* and up to 10% in *N. arcticum*, and removal of visible cephalodia reduced their N_2 fixation activity to 10–20% of control values (Sundberg *et al.*, 2001). In both species, cephalodia were smaller in marginal areas than in the central parts of the thallus. Cephalodia were deeper embedded in *N. arcticum* compared to *P. aphthosa*, but were easy to separate from underlying hyphae in both species. These underlying hyphae apparently remain relatively undisturbed after this procedure, because there have been no signs of necrosis or parasitic infections in these 'spots' or any other obvious signs of disturbance such as drastically altered respiration (L. Dahlman, B. Sundberg & K. Palmqvist, unpublished).

All transplantation frames were irrigated approx. twice a week with 8 l m^{-2} of artificial rainwater (Tamm, 1953), but with the phosphorus addition of the recipe excluded. At each irrigation event, thalli of each N addition treatment received 20 mg N m^{-2} of the specific N sources (Table 1), and these N compounds contained 20 atom% ^{15}N. The N source was dissolved in the artificial rainwater. Irrigation started at around 09:00 h and lasted for 5 min per frame. The lichens were first wetted with a small amount of the water-N solution to hydrate all thalli, thereafter irrigation continued when the lichens were wet and fully expanded. The control and $-N_{fix}$ thalli received only rainwater. The lichens were irrigated 25 times, thus corresponding to 200 mm (200 l m^{-2}) precipitation and a total addition of 500 mg N m^{-2} (5 kg N ha^{-1}, 1 kg ^{15}N ha^{-1}). Natural precipitation during the same period at the site was 97 mm. Nonwatered thalli used in a parallel study were wet and metabolically active for 11:00 h while the thalli of this study were wet and active for 15:00 h (K. Palmqvist & L. Dahlman, unpublished). Deposition of NH_4^+ plus NO_3^- in this area of Sweden is 500 mg N m^{-2} y^{-1} (Lövblad *et al.*, 1992), so on a yearly basis the added N was four times higher than this background.

Separation of new and old tissue

In the following analyses of lichen components and N assimilation (see below), five thalli were randomly selected from each species and manipulation treatment, and three thalli of each species were selected from the controls. All 10 thalli of each treatment and species were included in the growth analysis.

Digital photos were taken of each individual at the start of the experiment, in the middle of the period and immediately before harvest. By comparing these photos, using Adobe Photoshop®™ computer software (Adobe Systems Inc., San Jose, CA, USA), it was possible to identify thallus tissue formed during the course of the experiment. The newly formed (marginal; M) areas could then be dissected from the older parts (main body; B), and these were analysed separately in the following assays. All samples were freeze-dried, milled to a powder, divided into smaller subsamples for the different assays, and stored at −20°C in sealed vials until further analysis.

Nitrogen analyses

An approx. 2 mg freeze dried and milled lichen sample was analysed on a carbon-nitrogen analyser (Europa Scientific, ANCA-NT system, Solid/Liquids preparation module, Crewe, Cheshire, UK) coupled to a Europa 20–20 Isotopic Ratio Mass Spectrometer (IRMS), also supplied by Europa Scientific giving the ^{14}N to ^{15}N ratio and quantifying total N content according to Ohlsson & Wallmark (1999).

Cellular markers

Chlorophyll *a* was used as a unique marker for the photobiont cells (Raven, 1992) and the fungal cell wall component chitin was used to assess fungal biomass as in Palmqvist *et al.* (1998), and Sundberg *et al.* (2001). Ergosterol is the dominant sterol in most fungi, including lichens (Elix, 1996), being the principal constituent of the fungal plasma membrane (Weete, 1973). This compound has been used as a marker for the metabolic activity of fungi in mycorrhizal associations with plants (Ekblad & Näsholm, 1996), and has been related to steady-state respiration rates in lichens (Sundberg *et al.*, 1999).

Chlorophyll analysis

Between 5 and 10 mg of lichen powder was extracted in $MgCO_3$ saturated dimethyl sulphoxide (60°C for 40 min). After centrifugation (*c.* 20 000 ***g***, 5 min), the extracted Chl was quantified in the supernatant as detailed in Palmqvist & Sundberg (2001).

Ergosterol analysis

Ergosterol was measured as described in detail in Dahlman *et al.* (2001). Briefly, 10 mg of lichen powder was mixed with 1 ml ethanol (99.5%), intensively shaken on a Vortex, and incubated on a shaker in darkness for 30 min. After centrifugation (*c.* 20 000 ***g***, 15 min), ergosterol was determined in the supernatant by HPLC (Waters, Milford, MA, USA), using an ODS-ultra sphere column (250 mm × 4.6 mm; particle size 5 μm) as separator and isocratic elution with methanol as the mobile phase. Ergosterol absorption at 280 nm was measured with a UV detector. Data were corrected for an extraction yield of 80% (cf. Dahlman *et al.*, 2001).

Chitin analysis

Chitin was measured in the pellet remaining after ergosterol extraction (Dahlman *et al.*, 2001). Briefly, the sample was treated with 0.2 M NaOH to remove proteins and amino acids that could interfere with the HPLC analysis of glucosamine. Acid hydrolysis (6 M HCl) was used to release the glucosamine residues, which were converted to fluorescent derivatives by

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treatment with 9-fluorenylmethylchloroformate. The derivatives were subsequently analysed by RP-HPLC, using gradient elution (Ekblad & Näsholm, 1996). Data were corrected for an extraction yield of 83%.

Growth analysis

Weight and area changes of each individual thallus were obtained by subtracting their initial d. wt or area from their respective d. wt or area at harvest. Growth rates, that is, $\Delta g\ g^{-1}$ or $\Delta m^2\ m^{-2}$, were obtained by dividing the biomass or area changes of each thallus with its average dry weight or area between start and harvest. Individual changes in thallus specific weight (TSW; $g\ m^{-2}$) were obtained by subtracting TSW_{start} from $TSW_{harvest}$ and dividing this difference with TSW_{start}.

Nitrogen assimilation

Apoplastic and symplastic N was not separated in the analysis of thallus N concentrations, so true N assimilation could not be deduced. Therefore, N acquired from the additions, as calculated from Eqn 1, will be denoted N uptake.

$$\text{N uptake} = [[(^{15}N_s - {}^{15}N_c) \times (totN_s)]/0.2] \qquad \text{Eqn 1}$$

(where $^{15}N_s$, the At% of ^{15}N of the respective sample; $^{15}N_c$, average At% of ^{15}N of the control thalli; $totN_s$, the total $^{14}N + {}^{15}N$ concentration ($g\ g^{-1}$ d. wt) of the sample; and the value 0.2 corrects for the fraction of labelled ^{15}N in the N additions (20%) (see above)). An area based N uptake was obtained by dividing the weight based N uptake with the thallus specific weight of the sample ($g\ m^{-2}$). Marginal (M) and main body (B) parts were compared with the same part of the controls. For *N. arcticum* (control) the average At% ^{15}N was 0.3671 in B, and 0.3681 in M. For *P. aphthosa* (control) the average At% ^{15}N was 0.3660 in B, and 0.3667 in M.

Statistics

The effects of the different treatments were tested within species by one-way ANOVA using a statistical package (SPSS Inc., Chicago, IL, USA). Linear regressions were made using Sigma Plot (Sigma Plot 5.0, SPSS Inc, Chicago, IL, USA). Further details are given in the figure and table legends.

Results

Nitrogen uptake

Total N in the thalli was generally not affected by the different treatments, with average N concentrations being similar in all groups (Fig. 1c–d). Total N concentrations were higher in *P. aphthosa* compared to *N. arcticum*, varying between 3.0 and 4.7 $g\ m^{-2}$ in the former species and between 1.9 and 3.1 $g\ m^{-2}$

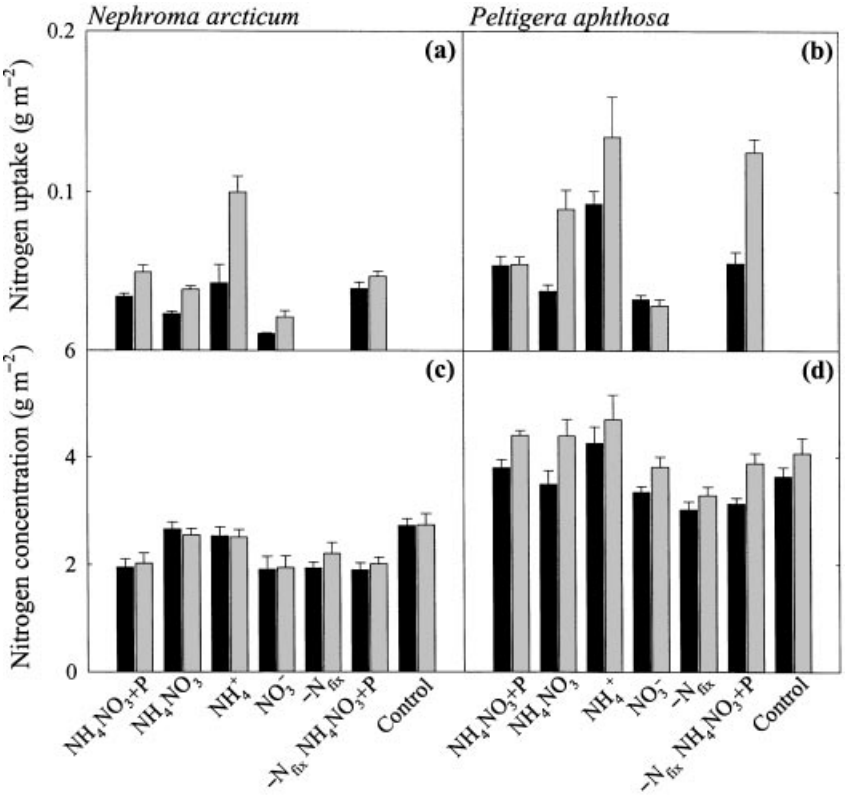

Fig. 1 Mean N uptake in the margins (grey columns; a,b) and main body (black columns; a,b), and mean total N concentrations in relation to thallus area at harvest for margins (grey columns; c,d) and main thallus body (black columns; c,d) for the different treatment groups (see Table 1), of *Nephroma arcticum* (a,c) and *Peltigera aphthosa* (b,d). Error bars represent ± 1 SE for $n = 5$, except for control were $n = 3$.

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in the latter. These thallus N concentrations are in full agreement with previous studies of these species (Palmqvist *et al.*, 1998; Sundberg *et al.*, 2001). The lichens had taken up between 11 and 134 mg N m^{-2} (Fig. 1a–b) of the 500 mg N m^{-2} that was added during the transplantation period. The N taken up from the additions constituted 0.6–4.0% of their total thallus N. In both species, and irrespective of treatment, more of the added N, in relation to total thallus N, was found in the margins compared to main body. In *N. arcticum* margins, 1–4% of the total N could be assigned to the added N, while in the main body 0.6–2% of the N came from the N additions. In *P. aphthosa*, 1–3% of the marginal N, and 1–2% of the main body N, were derived from the externally added N. Uptake was highest in NH_4^+-exposed thalli, intermediate for NH_4NO_3 treated thalli and lowest for NO_3-treated thalli (Fig. 1a–b). The addition of phosphorus did not significantly affect the uptake of NH_4NO_3, and the uptake of NH_4NO_3 was the same in thalli without cephalodia as for thalli with cephalodia (Fig. 1a–b).

Growth

On average, *N. arcticum* thalli increased in weight by *c.* 0.25 g g^{-1} during the 3 month experiment, and the weight-change was not affected by the treatments (Fig. 2a). For *P. aphthosa* the average weight-gain was *c.* 0.4 g g^{-1} of the control and N exposed thalli (Fig. 2b). In this species, weight gain was lower, *c.* 0.3 g g^{-1}, in both treatment groups where cephalodia had been removed.

Thallus expansion rate was significantly reduced in some of the treatment groups. In *N. arcticum*, the N-exposed thalli had increased by *c.* 0.2 m^2 m^{-2}, while the control thalli had increased with 0.4 m^2 m^{-2}. Thalli without cephalodia also increased less in area compared to the control (Fig. 2c). In *P. aphthosa*, the N additions did not affect area expansion, while thalli without cephalodia had a reduced thallus expansion compared to the others (Fig. 2d). In this species, average thallus expansion of control and N exposed thalli was 0.35 m^2 m^{-2} and 0.20 m^2 m^{-2} for those without cephalodia. An altered growth pattern of N-exposed *N. arcticum* was also evidenced by an increased thallus specific weight during the experiment. All N exposed thalli, including $-N_{fix}NH_4NO_3$ + P, had increased in TSW with 10–20%, while control thalli and $-N_{fix}$ had increased with *c.* 4% in TSW (Fig. 2e). For *P. aphthosa* there were no significant differences in TSW between the treatments with an average increase in TSW of *c.* 10% (Fig. 2f).

Cellular markers

Chitin concentrations ranged between 1.9 and 3.2 g m^{-2} in *N. arcticum*, and between 2.5 and 6.1 g m^{-2} in *P. aphthosa*, without significant treatment effects (Table 2). Consistent with a previous study of the same species (Sundberg *et al.*, 2001), chitin concentrations were lower in the margin compared to main body, particularly so in *P. aphthosa* (Table 2).

Overall, *P. aphthosa* had a twofold higher Chlorophyll *a* concentration compared to *N. arcticum*, the former species ranging between 180 and 260 mg m^{-2} in the main body and 160–280 mg m^{-2} in the margins, and the latter species ranging between 80 and 140 mg m^{-2} in the main body and 90–160 mg m^{-2} in the margins (Table 2).

The Chlorophyll *a* to *b* ratio was in the same range for both species, being 2.5–3.1 in the margins and 3.2–4.0 in the main body for *N. arcticum*, and 2.6–3.0 and 3.0–3.8 in *P. aphthosa* (Table 2). The higher Chlorophyll *a* to *b* ratio in main thallus body compared to margin can be attributed to the higher concentration of cephalodial *Nostoc* cells, which lack Chlorophyll *b*, in the centre of thalli (Sundberg *et al.*, 2001).

Ergosterol was more variable in *N. arcticum* than in *P. aphthosa*, ranging between 80 and 180 mg m^{-2} in the main body and between 90 and 240 mg m^{-2} in the margins (Table 2). In *P. aphthosa*, ergosterol ranged between 90 and 110 mg m^{-2} in the main body and between 130 and 180 mg m^{-2} in the margins. The NH_4NO_3 + P, $-N_{fix}$ and $-N_{fix}$ NH_4NO_3 + P treated thalli of *N. arcticum* showed higher ergosterol concentrations compared to control thalli (Table 2). In *P. aphthosa* there were no significant differences in ergosterol concentration between the treatments (Table 2).

Discussion

Nitrogen uptake

The primary aim of the current study was to assess uptake of externally added N by the two tripartite lichen species *Peltigera aphthosa* and *Nephroma arcticum*. Elevated levels of ^{15}N in all N exposed thalli and of both species showed that the lichens absorbed the added N, and the isotopic data showed an N uptake of 11–134 mg m^{-2} during the treatment period (Fig. 1a–b). Recalculated, this amounts to an uptake of 2–27% of the 500 mg N m^{-2} that was supplied during the experiment. Thallus N concentrations were not significantly changed by the N exposure (Fig. 1c–d), since the absorbed N contributed only marginally (1–4%) to the total thallus N concentrations. These results support the observations of a previous study of the same lichen species, where the uptake of added N appeared to be low (Sundberg *et al.*, 2001). Such a low absorption of added N is in clear contrast to the behaviour of an Antarctic green algal lichen (*Usnea sphacelata*), which absorbed *c.* 90% of both the NH_4^+ and the NO_3^- of accumulated wet deposition in the form of snow (Crittenden, 1998). However, the data presented here agree with data obtained for another green algal lichen (*Cladonia portentosa*) (Hyvärinen & Crittenden, 1998). In the latter study, lichen N uptake was significantly lower than the N load in regions where deposition is high. On the other hand N uptake matched deposition in northern Scotland where N deposition

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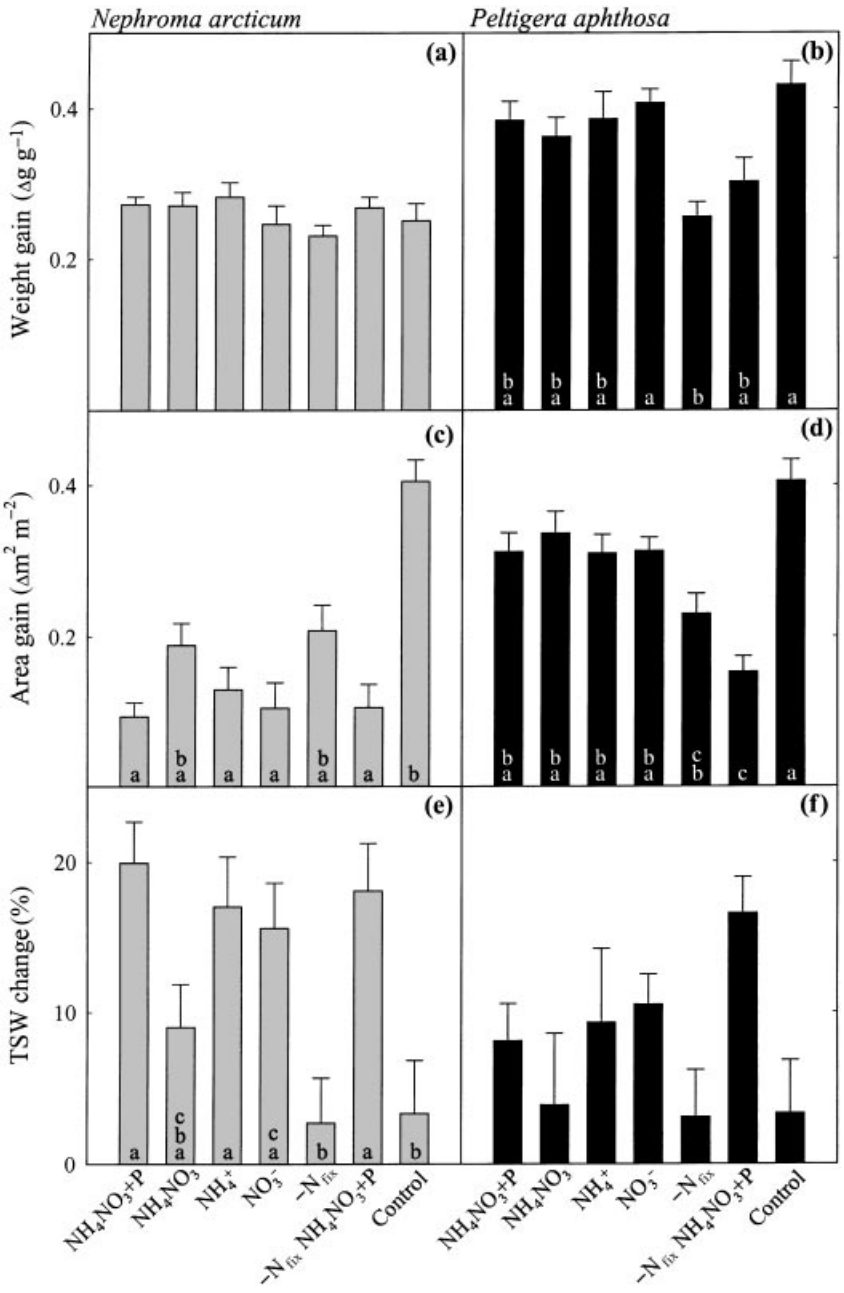

Fig. 2 Mean d. wt (a,b), area (c,d), and thallus specific weight (TSW) changes (e,f) for the different treatment groups (see Table 1), of *Nephroma arcticum* (grey columns; a,c,e) and *Peltigera aphthosa* (black columns; b,d,f). The lichens were wet and metabolically active for *c.* 15:00 h during the treatment period, and received a total irradiance of 170 mol photons m^{-2} when they were wet (K. Palmqvist & L. Dahlman, unpublished). Bars with different letters are significantly different ($P < 0.05$), error bars represent ± 1 SE for $n = 10$. ANOVA was made within species.

is low. In view of the results presented here, it then appears that at least some lichens have an ability to refrain from assimilation of excessive N and maintain a balanced tissue N concentration.

Nitrogen peferences

One striking result from our study was that NH_4^+ was absorbed to a greater extent than NO_3^- (Fig. 1a–b). In general, ^{15}N levels of NH_4^+ treated thalli were *c.* four times higher than for NO_3^- treated thalli. The extent of NH_4^+ uptake was 100 mg N m^{-2} for *N. arcticum* and 134 mg N m^{-2} for *P. aphthosa*, respectively, while corresponding figures for NO_3^- treated thalli were 24 and 32 mg N m^{-2}. The difference in recovery of added NH_4^+–N and NO_3^-–N was especially obvious in the margins of both species. This behaviour agrees with the observations for *Usnea sphacelata* where NO_3^- assimilation was shown to be oxygen dependent while NH_4^+ assimilation was little affected by O_2 deprivation (Crittenden, 1996). This also reflects the increased energy requirement of NO_3^- reduction as opposed to NH_4^+ assimilation (cf. Chapin *et al.*, 1987; Raven *et al.*, 1992). Preferential uptake of NH_4^+

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Table 2 Chlorophyll, chitin and ergosterol measurements

Species/ Treatment	Thallus part		Component			
			Chl *a* (mg m^{-2})	Chl *a* : *b* (g : g^{-1} ratio)	Chitin (g m^{-2})	Ergosterol (mg m^{-2})
Nephroma arcticum	Control	B	90 ± 20	3.2 ± 0.0	2.8 ± 0.1	80 ± 10^{A}
		M	130 ± 10	3.0 ± 0.0	2.4 ± 0.2	*120 ± 10*
	NH_4NO_3 + P	B	80 ± 10	3.6 ± 0.2	2.3 ± 0.1	150 ± 10^{AB}
		M	120 ± 20	3.1 ± 0.1	1.9 ± 0.2	*160 ± 20*
	NH_4NO_3	B	130 ± 20	3.9 ± 0.2	3.2 ± 0.2	170 ± 10^{B}
		M	150 ± 20	3.0 ± 0.3	2.5 ± 0.1	*120 ± 20*
	NH_4^+	B	120 ± 20	4.0 ± 0.3	2.6 ± 0.3	150 ± 10^{AB}
		M	160 ± 10	3.2 ± 0.2	2.1 ± 0.2	*190 ± 20*
	NO_3^-	B	80 ± 10	3.4 ± 0.5	2.2 ± 0.2	130 ± 10^{AB}
		M	90 ± 10	2.6 ± 0.2	1.7 ± 0.3	*90 ± 30*
	$-N_{fix}$	B	140 ± 30	3.7 ± 0.6	2.3 ± 0.3	180 ± 20^{B}
		M	140 ± 30	2.5 ± 0.3	2.2 ± 0.2	*200 ± 30*
	$-N_{fix}NH_4NO_3$ + P	B	100 ± 20	3.6 ± 0.5	2.4 ± 0.2	170 ± 10^{B}
		M	100 ± 10	2.7 ± 0.3	1.8 ± 0.1	*240 ± 30*
Peltigera aphthosa	Control	B	230 ± 10	3.8 ± 0.2	6.1 ± 0.2	110 ± 20
		M	230 ± 10	2.9 ± 0.0	3.9 ± 0.3	*130 ± 30*
	NH_4NO_3 + P	B	210 ± 20	2.9 ± 0.2	5.6 ± 0.2	110 ± 10
		M	230 ± 30	2.9 ± 0.1	4.2 ± 0.4	*180 ± 10*
	NH_4NO_3	B	260 ± 60	3.9 ± 0.8	5.4 ± 0.2	100 ± 10
		M	230 ± 30	2.8 ± 0.3	3.9 ± 0.2	*150 ± 10*
	NH_4^-	B	190 ± 10	3.0 ± 0.2	5.9 ± 0.5	100 ± 10
		M	280 ± 30	3.1 ± 0.1	3.6 ± 1.0	*160 ± 20*
	NO_3^-	B	180 ± 20	3.1 ± 0.3	4.8 ± 0.2	100 ± 10
		M	190 ± 20	3.2 ± 0.4	2.5 ± 0.7	*140 ± 10*
	$-N_{fix}$	B	190 ± 30	3.2 ± 0.3	4.0 ± 1.1	100 ± 10
		M	180 ± 40	3.0 ± 0.7	3.3 ± 0.1	*150 ± 10*
	$-N_{fix}NH_4NO_3$ + P	B	180 ± 40	3.3 ± 0.4	5.0 ± 0.3	90 ± 20
		M	160 ± 20	2.6 ± 0.3	3.4 ± 0.1	*140 ± 20*

The table shows chlorophyll *a*, chitin and ergosterol concentrations and Chlorophyll *a : b* ratios in relation to thallus area for the thalli harvested in September. Margins (M) were cut from the thallus main body (B) as detailed in the Materials and Methods section. Values for B and M are the mean ± 1 SE of $n = 5$, except for the control thalli where $n = 3$. ANOVA was made within species, testing M and B separately. Significant differences are indicated by different letters ($P < 0.05$).

is a further common phenomenon also in plants, as reported by several authors (Marschner *et al.*, 1991; Kronzucker *et al.*, 1997). In addition, NH_4^+ assimilation is also faster than NO_3^- uptake in most fungi (Smith & Read, 1997). Moreover a higher recovery of NH_4^+ than of NO_3^- in the lichens could also be due to the fact that NH_4^+, as a cation, is adsorbed to the negatively charged groups present on hyphal cell walls.

The highest tissue concentrations of added N amounted to 0.1 g N m^{-2} (see above), while total N concentrations in newly made tissue were significantly higher, 2 g m^{-2} for *N. arcticum* and 4 g m^{-2} for *P. aphthosa* (Fig. 1). This implies that the dominant part of the N required for making new tissue must have been provided by some other source than the added N, for example, possibly through their own N_2-fixation. The lichens were naturally wet for *c.* 11:00 h during the transplantation period, when they presumably had both photosynthetic and N_2-fixation activity (Palmqvist & Sundberg, 2000). Irrigation events resulted in *c.* 400 h of additional wet active time (see Methods), when their N_2-fixation activity might have been partially inhibited (Rai *et al.*, 1981). Approximately two thirds of their metabolically active periods hence occurred when they were wetted by natural precipitation. So, even if their N_2-fixation was partially inhibited during the active periods of the N irrigation events, this was probably not the case when they were 'naturally activated'. It is still surprising that the irrigated thalli without cephalodia had not absorbed more of the added N (Fig. 1). This implies that continuous removal of visible cephalodia was not sufficient to fully inhibit N_2-fixation. Indeed as much as 10–20% of their initial N_2-fixation activity may have remained after cephalodial removal (Sundberg *et al.*, 2001). Moreover, several thalli developed new cephalodia during the course of the experiment, so during active periods between the irrigation events and before removal of these, the thalli had the opportunity to fix N_2. In addition, the background deposition of N may also have contributed to the N budget of the N-starved thalli.

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Thallus nitrogen allocation

The highest concentration of labelled N was found in the margins of the thalli (Fig. 1a–b). However, an opposite pattern would be expected if added N had been assimilated equally across the thalli, since the most recently formed thallus parts were exposed to fewer fertilization treatments. Lichen growth coincides with wet periods (K. Palmqvist & L. Dahlman, unpublished), and because these occurred relatively evenly during the transplantation period, it might be assumed that thallus expansion was close to linear over time. There are two possible explanations for the finding that there was more labelled N in the margins. First, assimilated N might have been actively translocated from the main thallus body towards the growing margins. Alternatively, assimilation of externally added N might have been more active in the margins of the thallus compared to the main body. In support of the former hypothesis it has previously been found that phosphorus is reallocated from the basal zone to the growing apex of the lichen *Cladonia portentosa* (Hyvärinen & Crittenden, 2000), suggesting a symplastic movement of nutrients within lichens. These authors also put forward the hypothesis of re-circulation of nutrients within lichen thalli, allocating N and phosphorus from older, nonactive, parts towards more actively growing parts following a sink/source model. Our result lends some support to the existence of such a nutrient allocation mechanism in lichens.

The latter hypothesis of a larger assimilation of the added N in the more active margins of the thallus is supported by the fact that cephalodia are denser in the main body, and that heterocyst frequency is higher in central compared to marginal areas (Rai, 1988). In addition, the relative proportion of algal biomass in relation to mycobiont biomass was higher in the margins (Table 2, Sundberg *et al.*, 2001). In this case, one might speculate that the photobiont is more prone to absorb N from an external source rather than awaiting export from cephalodia and through the fungus. This idea is supported by the higher Chlorophyll *a* concentration in the thallus margin of NH_4^+-fertilized thalli of both species (Table 2).

Effects on growth

The most obvious effect of the nitrogen stress was on the area gain of both lichen species (Fig. 2c–d). This is in accordance with earlier studies (Sundberg *et al.*, 2001) showing that lichen expansion is affected by alterations in N supply. Also in accordance with earlier studies, weight gain was not affected by N additions, although it was decreased by removal of cephalodia, at least in *P. aphthosa* (Fig. 2a–b). The resulting increase in specific weight of the lichen thalli (Fig. 2e–f) may be due to an increased production of carbon storage substances (cf. Sundberg *et al.*, 2001) compounds that may constitute 5–10% of the lichen thallus weight (cf. Palmqvist, 2000).

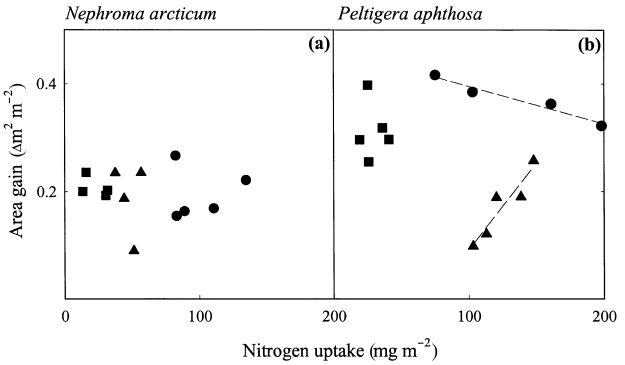

Fig. 3 The relationship between thallus expansion and N uptake in the thallus margin of *Nephroma arcticum* (a) and *Peltigera aphthosa* (b). Symbols represent the different N manipulations: NO_3^- (squares); $-N_{fix}NH_4NO_3$ + P (triangles); NH_4^+ (circles). Each symbol represents an individual thallus and N uptake data reflects those of the margins of the thalli. Regression lines are given when the slope value of the correlation between area gain and N uptake was significantly different from zero. *P. aphthosa* $-N_{fix}$ NH_4NO_3 + P($y = -0.23 + 0.0032x$; $r^2 = 0.93$), and NH_4^+($y = 0.46 - 0.0007x$; $r^2 = 0.84$), $P < 0.05$ for both.

Because both the addition of N, as well as N deprivation, caused a similar growth response it can be suggested that thallus expansion is tightly regulated by thallus N status. For *P. aphthosa*, the inhibition of thallus expansion was well correlated with N uptake, giving a decreased area gain with increased N uptake (Fig. 3b). The opposite pattern was, however, found for thalli where cephalodia had been removed and N added. In *N. arcticum* there was no correlation between the inhibited growth and N uptake for the different treatments (Fig. 3a). This might possibly be related to the lower N uptake in this species.

Effects on cellular markers

In spite of the clear growth response towards the two forms of N stress, there was no significant alteration in chitin to Chlorophyll *a* ratios in the treated thalli. However, the ergosterol concentration was significantly increased in some of the N stressed thalli of *N. arcticum* (Table 2). Increased ergosterol concentrations suggest a larger fraction of living fungal biomass in the thalli and therefore an increased respiratory load and by that a higher demand for photosynthetically fixed carbon for these thalli (Sundberg *et al.*, 1999). As a result, the growth patterns of these thalli might be altered, as was indeed also the case (Fig. 2). Sundberg *et al.* (2001) also found a tendency of increased ergosterol concentration concomitant with reduced area expansion in N manipulated *N. arcticum* thalli, again in accordance with our study.

Conclusion

The present study shows, through the use of ^{15}N labelled N sources, that two common foliose, tripartite, and N_2-fixing

lichen species absorb NH_4^+ and NO_3^- from added N. The fraction of added N recovered in lichen thalli was higher for NH_4^+ than for NO_3^-. Further, a relatively high concentration of labelled N in margins of the thalli suggest that the lichens reallocated absorbed N from older parts to actively growing parts or that N uptake from external sources were higher in the margins of the thallus, were photobiont concentrations are relatively higher. An altered biomass allocation between the bionts was found in *N. arcticum*, as evidenced by an increased concentration of ergosterol in N stressed thalli, suggesting a higher respiratory load in these. In spite of the low N uptake and the small effect on thallus N concentrations, a reduced rate of thallus expansion was recorded for thalli exposed to both types of N stress, that is, both addition and removal of N. Clearly, the mechanism through which N affects thallus expansion rate in lichens merits further studies.

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