# Nitrogen fixation in the lichen *Stereocaulon paschale*

by

Kerstin Huss-Danell

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*Abstract:* The thesis is a summary and discussion of six papers.

The purpose of the investigation was to study the influence of (i) environmental factors and (ii) the physiological condition of the thallus on nitrogen fixation in the lichen *Stereocaulon paschale* (L.) Fr. The nitrogen input to the site, a sparsely stocked pine forest in northern Sweden, was also studied. Nitrogen fixation (nitrogenase activity) was measured as acetylene reduction both in the field and in the laboratory. Usually intact lichen thalli were used, but also excised cephalodia were studied.

All nitrogenase activity was located in the external cephalodia containing the blue-green alga *Stigonema* sp. There was always a reduction in nitrogenase activity when the cephalodia were quantitatively excised from the thallus. Moisture was found to be the most important environmental factor in the field during the snow free part of the year. At the site, with 14 % of the ground covered by *S. paschale*, the yearly nitrogen fixation was estimated to *c.* 0.1 g nitrogen per $m^2$. The lichen thalli could withstand several months in a very dry condition and at a low temperature without significant decrease in nitrogenase activity. A higher capacity for nitrogenase activity was found in lichen thalli collected from bare ground than in thalli collected under the snow. The light conditions before as well as during the nitrogenase activity measurements affected the nitrogenase activity. Thalli incubated with acetylene in the dark had only *c.* two thirds of their activities in the light. Lichen thalli pretreated in the light showed increased nitrogenase activities, probably due to raised content of carbohydrates available for nitrogenase activity. The necessary energy for nitrogenase activity is supplied by either oxidative phosphorylation or photophosphorylation.

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## 1. List of papers

This thesis is a summary and discussion of the following papers, which will be referred to by their Roman numerals.

I. Huss-Danell, K. 1977. Nitrogen fixation by *Stereocaulon paschale* under field conditions. - Can. J. Bot. 55:585-592.

II. Hällgren, J.-E. & Huss, K. 1975. Effects of $SO_2$ on photosynthesis and nitrogen fixation. - Physiol. Plant. 34:171-176.

III. Huss-Danell, K. 1977. Nitrogenase activity in the lichen *Stereocaulon paschale:* recovery after dry storage. - Physiol. Plant. 41:158-161.

IV. Huss-Danell, K. 1978. Seasonal variation in the capacity for nitrogenase activity in the lichen *Stereocaulon paschale*. - New Phytol. 81:89-98.

V. Huss-Danell, K. 1979. The influence of light and oxygen on nitrogenase activity in the lichen *Stereocaulon paschale*.

VI. Huss-Danell, K. 1979. The cephalodia and their nitrogenase activity in the lichen *Stereocaulon paschale*.

## 2. Introduction

Biological nitrogen fixation, *i.e.* reduction of $N_2$ to $NH_3$ with the aid of the enzyme nitrogenase, is restricted to certain bacteria and blue-green algae. In lichens nitrogen fixation is due to presence of a nitrogenase containing blue-green alga in the thallus. Henriksson (1951) showed that the isolated *Nostoc* of *Collema tenax* could fix nitrogen when grown in culture, but the first definitive demonstration that intact thalli fix nitrogen was by Bond and Scott (1955) who used $^{15}N_2$ and tested *Collema granosum* and *Leptogium lichenoides*. Later, a number of both field and laboratory studies on nitrogen fixation in lichens have been done; they are summarized by Millbank (1977).

*Stereocaulon paschale* (L.) Fr. is a terricolous nitrogen-fixing lichen species with a circumpolar distribution (Lamb 1951). It is common in the coniferous forests on alluvial sediments along the rivers in northern Sweden. Nitrogen fixation in *S. paschale* from northern parts of Finland and Canada has been studied, mainly in the field, by Kallio (1976) and Crittenden and Kershaw (1978). At the site for this study *S. paschale* lives in a habitat where the occurrence and nitrogen-fixing activity of free-living micro-organisms is very small (Sundström and Huss 1975) and where no other known symbiotic nitrogen-fixing organisms are to be found. The site is a sparsely stocked pine (*Pinus silvestris*) forest with the ground covered by a lichen mat with scattered clones of *Calluna vulgaris*. The lichen mat is dominated by *Cladonia* spp., but *c.* 14 % of the ground is covered by *S. paschale* (I).

The purpose of this investigation was to study the influence of (1) environmental factors and (2) the physiological condition of the thallus on nitrogen fixation in the lichen *S. paschale*. The nitrogen input to the site by nitrogen fixation in this species was also studied.

## 3. The lichen *Stereocaulon paschale*

The fruticose thallus of the lichen *S. paschale* consists of three components; a fungus (Ascomycetes), a green alga (*Trebouxia* sp.) and a blue-green alga (*Stigonema* sp.). *Stigonema* is located in external cephalodia. The cells of *Stigonema* are of two major types, vegetative cells and heterocysts (VI). In studies on free-living blue-green algae almost all data show that the heterocysts contain the enzyme nitrogenase and are the site for nitrogen fixation (Stewart 1977). The heterocysts have an incomplete photosynthetic apparatus with the oxygen evolving photosystem II and the $CO_2$-fixing enzyme ribulose-1,5-bisphosphate carboxylase lacking (Stewart 1977). The photosynthesizing vegetative cells export carbohydrates to the nitrogen-fixing heterocysts (Wolk 1968). These biochemical differences between heterocysts and vegetative cells have not yet been reported for blue-green algae *in situ* in lichens, but there is a relation between the frequency of heterocysts and nitrogenase activity in *Nostoc* of *Peltigera* spp. (Hitch and Millbank 1975, Stewart and Rowell 1977).

In *S. paschale* all nitrogenase activity was located in the cephalodia (VI). Excised cephalodia showed constant nitrogenase activity for at least two days in the light (VI) and responded to alternating light/dark periods in the same way as did intact thalli (VI, V). However, there was always a reduction in nitrogenase activity when the cephalodia were excised from the thalli (VI). The need for an intact thallus was further demonstrated by the loss of activity upon cutting the thallus in pieces without rupturing the connection between the cephalodia and the main thallus (VI).

At present there is no possibility to grow lichens in the laboratory in order to harvest enough of lichen material for the present type of studies. It is, therefore, necessary to collect the experimental material from the field. Until experimental use the thalli can then be kept either physiologically active in a growth chamber (Kershaw and Millbank 1969), or in a physiologically almost inactive condition (III). *S. paschale* could withstand several months in a very dry condition and at a low temperature without significant changes in nitrogenase activity. This was the pre-requisi-

te for the storage technique used (III). Also in *Collema tuniforme* and *Peltigera rufescens* the nitrogenase activity recovered after dry periods (Henriksson and Simu 1971). In *Peltigera polydactyla* the nitrogenase activity was clearly negatively affected already after one week of dry storage (Kershaw and Dzikowski 1977).

## 4. Measurements of the nitrogenase (nitrogen fixation) activity

The nitrogenase activity in *S. paschale* has been measured as acetylene reduction throughout this study. The technique is based upon the discovery that acetylene is one of the substrates that can be reduced by the enzyme nitrogenase (Dilworth 1966, Schöllhorn and Burris 1966). For each mol $C_2H_2$ that is reduced to $C_2H_4$ two electrons are transferred, while six electrons are required for the reduction of one mol $N_2$ to two mol $NH_3$. This results in a theoretical molar ratio of 3:1 for $C_2H_4$:2 $NH_3$ (Burris 1974). When the partial pressure of acetylene in the gas phase is high enough all electrons in the nitrogenase reaction are used for acetylene reduction, but when $N_2$ is the substrate the formation of hydrogen gas from protons competes with $N_2$ reduction. This competition by hydrogen formation results in molar ratios different from the theoretical one. The true molar ratio has to be determined by comparing the $C_2H_2$ reduction with $^{15}N_2$ reduction (Burris 1974). For *S. paschale* no such comparison is available. To convert the nitrogenase activities into g N fixed the theoretical molar ratio was used (I), although it probably caused a small overestimation. Except for the studies on the influence of oxygen on the nitrogenase activity (V), air has been used for incubations with acetylene. A partial pressure of 10 kPa $C_2H_2$ was tested to be more than enough for maximal $C_2H_2$ reduction rates, and has been used throughout this study.

## 5. Influence of environmental factors on nitrogenase activity

### 5.1. Light

In the field the thalli of *S. paschale* were studied under a wide variety of irradiances, but no relationship between nitrogenase activity and irra-

diance was seen (I). At high irradiances the thalli in the field were too dry for nitrogenase activity. In the laboratory a rapid effect of light upon nitrogenase activity in *S. paschale* was seen (V, VI). When light activated wet thalli were incubated with $C_2H_2$ in the dark the activity dropped to *c.* two thirds of the activity in the light.

Light has an important role for nitrogenase activity in *S. paschale*, probably by providing photosynthetic products. After pretreatments of the thalli under conditions where photosynthesis was possible, the nitrogenase activity increased (III, IV, V). A pretreatment in the light for 12-36 hours was also useful for restoring the original nitrogenase activity in thalli stored for a long time (III).

An effect of daylength on the nitrogenase activity was also observed (IV). This can be explained by alteration of the amount of available photosynthetic products. Long days together with short nights permit the thalli to synthesize more assimilates than are used up during the dark period. The importance of daylength for nitrogenase activity has also been shown for *Peltigera* spp. (MacFarlane *et al.* 1976, Kershaw *et al.* 1977).

## 5.2. Moisture

A relation between the nitrogenase activity and the water content of the thalli was demonstrated in the field study (I). It has also been described from laboratory studies on *S. paschale* (Kallio 1973) and other lichen species (see Millbank 1977). For *S. paschale* from the present site a water content of 0.75-0.80 $g \cdot g^{-1}$ dry weight was a threshold value for nitrogenase activity (I). Above this water content the nitrogenase activity increased and 1.5-1.8 $g \cdot g^{-1}$ dry weight was needed for full activity (Huss-Danell, unpublished).

The uptake and loss of water from a lichen thallus is largely a physical process (Blum 1973). *S. paschale* can gain water from moist air, although very slowly (I). Uptake from rain is a far more effective way of increasing

the water content of the thalli (I), and a relation between the water content of the thalli and the precipitation was established (I). Drying out is a rapid process when the air is dry. It is speeded up by wind, but is stopped during nights with a relative air humidity of 100 %.

*S. paschale* from the site is well adapted to withstand long periods in a very dry condition, at least at a low temperature (III). In the field *S. paschale* seldom remains in a very dry condition more than a few days or weeks before the thalli are wetted by precipitation. The very dry condition often occurs at high day temperatures and low, near freezing, night temperatures.

5.3. Temperature

Being an enzymatic process, the nitrogenase activity in lichens typically respond to the incubation temperature and an optimum at about 20-30 °C is generally seen (MacFarlane and Kershaw 1977, Millbank 1977). In the field low activities were found in *S. paschale* when the temperature was low (I). At high temperatures in the field the thalli had generally already dried out and were therefore inactive. In the laboratory studies 15 °C was used during pretreatments and nitrogenase activity incubations since this temperature was common in the field when the thalli were wet. The obtained nitrogenase activities (III-VI) were therefore not necessarily maximal.

Indirect effects of temperature on nitrogenase activity were also seen. When *S. paschale* was exposed to night temperatures below freezing subsequent measurements in the light at 15 °C showed increased rather than decreased nitrogenase activities (IV). A possible explanation was the expectedly low respiration rates in the frozen thalli. Thereby the carbohydrates were not used up very much in the dark. The generally low respiration rates at low temperatures were also considered when the conditions were selected for the experiments with long time storage of the thalli (III). The thermal sensitivity of dry *S. paschale* has not been investigated with regard to nitrogenase activity. Recently, however, Kershaw and Smith (1978) showed that photosynthesis in *S. paschale* is severely affected after exposure of dry thalli to 35 °C and higher temperatures.

Because of the retarding effect on the respiration rate, and thereby preservation of carbohydrates, the low ground temperatures in winter could be expected to enable the thalli to have high nitrogenase activities as soon as moisture, light and temperature conditions turned favourable. That only low nitrogenase activities were found (I, IV) might depend on other physiological changes during the winter, *e.g.* damage to cells and membranes and lack of active enzymes.

### 5.4. Air pollutants

In *S. paschale* the nitrogenase activity was found to be more sensitive than photosynthesis to $NaHSO_3$ dissolved in buffered solutions (II). An effect of low pH alone on nitrogenase activity in *S. paschale* was also seen (II). The $NaHSO_3$ concentrations used correspond to $SO_2$ concentrations occurring in heavily polluted areas (II, Ferguson *et al.* 1978). A higher sensitivity of nitrogenase activity than of photosynthesis to air pollutants was reported for transplantation experiments with *S. paschale* and *Nephroma arcticum* (Kallio and Varheenmaa 1974), but the composition of the air pollutants was not known.

The action of $NaHSO_3$ on nitrogenase activity and photosynthesis was further investigated in comparative studies on the free-living blue-green alga *Anabaena cylindrica* (II). Besides of a pH effect alone, the decrease in nitrogenase activity was pH dependent. The mechanism for the action of $NaHSO_3$ was not fully understood, but a rapid and direct effect on nitrogenase activity and not only an indirect effect via a lowered photosynthesis was found (II).

## 6. Role of photosynthesis and respiration

Photosynthesis is of great importance for nitrogenase activity in blue-green algae. The major sources of energy (ATP) for nitrogenase activity are photophosphorylation and oxidative phosphorylation, both processes occurring in heterocysts (Stewart 1977). Photosynthesis in vegetative cells

provides carbohydrates for subsequent metabolism and is indirectly involved in oxidative phosphorylation. In blue-green algae products of photosynthesis are also the source of reductant for nitrogenase activity by providing substrates which supply electrons in subsequent processes (Stewart 1977). Another role of photosynthesis in connection with nitrogen fixation is to provide carbon skeletons for amino acid metabolism. However, the formation of ammonia by nitrogen fixation is inhibited when $C_2H_2$ is the substrate for the nitrogenase (Burris 1974).

In *S. paschale* direct (V,VI) as well as indirect (III, IV, V) effects of light on nitrogenase activity were observed, as quoted above. The direct influence of light was seen also in short-time experiments (10 minutes alternating light/dark periods) (V), and a probable reason for the higher nitrogenase activity in the light was a supply of ATP by cyclic photophosphorylation. The indirect effects can be explained as a result of the build up of available photosynthetic products. The relation between nitrogenase activity and oxygen in the dark (V) suggested a large dependency on respiratory processes. In these experiments the thalli were pretreated in the light and were believed to have a large amount of available photosynthates.

When thalli of *S. paschale* were transferred to conditions favourable for nitrogenase activity a seasonal variation in the capacity for nitrogenase activity was seen (IV). The capacity is determined by the influence of the previous environmental conditions of the thalli, and the amount of carbohydrates available for nitrogenase activity explains a great deal of this capacity.

Dark respiratory processes not directly connected with nitrogenase activity may also influence the nitrogenase activity in *S. paschale*. A depletion of substrates was suggested to occur when the thalli were stored with a water content lower than the threshold value for nitrogenase activity (III) but, according to Kershaw and Smith (1978), high enough for dark respiration.

The nitrogen-fixing component of *S. paschale*, the *Stigonema* in the cephalodia, was able to produce photosynthetic products itself and to maintain a steady rate of nitrogenase activity (VI). The always reduced nitrogenase activity of excised cephalodia (VI) pointed to the possibility that photosynthates from the green alga in the thallus are transported to the cephalodia and utilized for nitrogenase activity there. However, such a cooperation of the green and blue-green phycobionts in *S. paschale* has yet to be proven.

## 7. Nitrogen input to the site by nitrogen fixation in *Stereocaulon paschale*

As found in this study, the various environmental factors and combinations of these affect the nitrogenase activity in the thalli of *S. paschale*. A considerable variation in nitrogenase activity therefore occurs between different thalli at one time and for a given thallus during a day and between days. For an estimate of the yearly nitrogen fixation in the field, with a certain amount of thalli, the influence of the prevailing as well as the previous environmental factors on the nitrogenase activity must be considered. In this study the water content of the thalli was the most important factor in the field during the snow free part of the year (I). In winter the thalli of *S. paschale* are not exposed to high irradiances because of the snow cover at the site (IV), and they are not expected to be very active in photosynthesis (cf. Barstow and Erbisch 1977, Kershaw and Smith 1978). Neither is nitrogenase activity probable under such conditions and negative results for *S. paschale* are in fact reported (IV, Alexander and Kallio 1976).

Thus the estimated nitrogen fixation at the site, 0.1 g N $m^{-2}$ during the period May - September (14 % of the ground covered by *S. paschale*) (I), will not need much addition to be valid for the whole year. At the site there are three possible ways for incorporation of the fixed nitrogen into the nitrogen cycle of the ecosystem; (1) by decomposition of thalli of *S. paschale*, (2) by leakage from dry thalli when rewetted by rain (cf. Millbank 1978), and (3) by animal (reindeer) comsumption.

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