Manganese as a site factor for epiphytic lichens

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Chapter 1

Introduction

Element contents of lichens

Cation metabolism in higher plants is fairly well studied.
The physiological function of essential ions as well as toxic effects of excess concentrations have been extensively investigated. In lichens, however, studies of cation metabolism have primarily been limited to the accumulation and toxic effects by transition metals, such as Cu, Pb, Cd or Ni, which are emitted to the atmosphere due to human activities (NASH 1989, GARTY 2001).
In low-polluted areas, cation uptake and resulting physiological effects have been scarcely studied in lichens (HAUCK 2003), cation sources for lichens are the substrate, precipitation and particles from dry deposition (FARMER et al. 1991, HAUCK 2000).

ULOTH (1861) showed that the foliose lichens *Evernia prunastri* had higher concentration of Fe on sandstone than on birch bark. Likewise, LOUNAMAA (1965) observed in southern Finland higher Fe concentrations in epiphytic lichens than in epilithic species. He also mentioned that the bark on which *Hypogymnia physodes* grew, was dark-coloured and was more fragile than bark in the immediate neighbourhood; which was not colonized by *H. physodes*. Therefore, chemical interaction presumably could have taken place in this case. Such interaction between lichens and substrate is known from epilithic lichens (JONES et al. 1981, WILSON & JONES 1984).

In the Harz Mountains, the occurence of metallophytic lichens on ferrous slag was studied by LANGE et al. (1963), focussing not only the plantsociological aspect, but also identifying the Fe and Cu content of selected lichen species. LANGE et al. (1963) and NOESKE et al. (1970) also and localized Fe and Cu within the thalli with the aid of histochemical methods. In Norway, SOLBERG (1967) studied the element content of some Norwegian lichens species; Mn concentrations of epiphytic species were included in this study.

SEAWARD (1974) studied the colonization of *Hypogymnia physodes* on trunks of *Fraxinus excelsior* with attached barbed wire. He reported that whereas the immediate surroundings of the barbed wire were devoid of lichens, whereas in a certain distance from the barbed wire thalli of *H. physodes* occured with elevated concentrations of Zn, Fe and Pb.

In Scotland, WILSON et al. (1984) observed crystals of Mn oxalate below the crustose lichen *Pertusaria corallina*, which were formed through the interaction of the mycobiont with the substrate.
DE BRUIN & HACKENITZ (1986) found a significant correlation between bark concentrations of Mn, Zn and Cd and thalli of *Parmelia sulcata*. DE BRUIN & HACKENITZ (1986) concluded from their results that *P. sulcata* took up these elements from the bark. SLOOF & WOLTERBEEK (1993) similarly suggested that *Lecanora conizaeoides* took up Cd, Zn and Mn from bark based on measurements of element concentrations in outer and inner bark as well as in lichen thalli.

A survey of studies dealing with lichens in heavy metal-enriched environments, especially of sites rich in Fe or Cu, was published by PURVIS (1996) and PURVIS & HALLS (1996).

BROWN (1987) pointed out the importance of separate analysis of intracellular and extracellularly bound element concentrations as well as of concentrations of ions trapped in extracellular particles. Information based merely on total concentrations is less usefull.

In the foliose lichen *Peltigera canina* from abandoned heavy metal mining sites, GOYAL et al. (1982) observed decreasing Mn concentrations from the rhizinae via fungal hyphae of the cortex and the medulla to the photobiont, whereas in low-polluted locations the lowest Mn concentrations were found in the rhizinae.

Since the beginning of the eighties, the element distribution among the lichen thallus was studied by means of X-ray microanalysis (EDAX), either in connection with a scanning electron microscope (SEM) or a transmission electron microscope (TEM).
ASTA, J. & J. P. GARREC (1980) studied the allocation of Ca, K, Mg and P in various lichen species. Ca was primarily allocated in the cortex. High concentrations of K, Mg and P were found in the algal layer.
GARTY et al. (1979) showed that *Caloplaca aurantia* contained particles that consisted of Fe, Cr, Ni, Mn and Zn and were associated with fungal hyphae.

Experimental studies of metal adsorption and toxicity in lichens

Numerous experimental studies were carried out to examine cation uptake and toxicity effects under laboratory conditions (GARTY 2001). However, long-term effects of metals were difficult to detect in such experiments, as prolonged culture of lichens is difficult (AHMADJIAN 1993).
Uptake and toxicity of Cd, Co, Pb, Hg, Ni and Ag metals in the lichen *Umbilicaria muhlenbergii* were studied by PUCKETT (1976). Cu, Hg and Ag produced an efflux of K, which increased with increasing concentration of Cu, Hg or Ag. K efflux was used as an indicator of membrane damage. Pertaining to Cd, Co, Pb and Ni, K efflux occurred abruptly when a certain threshold concentration was exceeded. Co, Pb, Cu and particularly Hg and Ag reduced photosynthetic ^{14}C fixation (PUCKETT 1976). Based on these results PUCKETT (1976) estimated increasing toxicity in the order Ni, Pb < Cd, Cu < Co < Ag, Hg for short exposures, Ni < Co, Pb ≤ Cu < Ag, Hg for prolonged exposures.

In a study of Ni accumulation in *Umbilicaria muhlenbergii*, NIEBOER et al. (1976) demonstrated that the metal adsorption is a passive, physical-chemical process. Ni adsorption was affected by temperature and pH. Consequently, it was concluded that the adsorption followed an ion-exchange model, as already shown by TUOMINEN (1967) with the adsorption of Sr in *Cladonia stellaris*, where ion bind to extracellular cation exchange sites, such as carboxyl and hydroxyl groups.

BECKETT et al. (1984a, b) studied cation uptake, which follows Michaelis Menten kinetics. Amounts of ions taken up intracellularly are substantially lower than amounts adsorbed at extracellular exchange sites. Intracellular uptake is presumably mediated by non-specific cation transporters. Moreover, BECKETT & BROWN (1984a) studied how Cd uptake was affected by the presence of other cations. Cd uptake showed most increasingly reduced in the sequence monovalent, class A metal < divalent class A metal < borderline metal < class B metal.

The classification of class A, class B and borderline metals refers to NIEBOER & RICHARDSON (1980). Class A metals favour oxygen bonds, whereas class B metals prefer to bind to sulphur and nitrogen and borderline metals mediate between class A and B metals. The classification is based on electron negativity, ionic radius and formal charge (AHRLAND et al. 1958, NIEBOER & RICHARDSON 1980). Among the class A metals are, e.g., K^+, Ca^{2+} and Mg^{2+}, which are generally not toxic to plants and are often required in larger quantities. High toxic metals such as Ag^+ and Hg^+ belong to the class B metals. Cu^{2+}, Co^{2+}, Ni^{2+}, Cr^{2+} and Mn^{2+} are examples classified for borderline metals. The latter are often essential trace elements, but are toxic in higher concentration.

Only few studies that analysed the effect of metals on epiphytic lichens have been published, so far. Zn adsorption kinetics was studied in the beard lichen *Usnea florida* (WAINWRIGHT & BECKETT 1975). RICHARDSON et al. (1985) sought for the probable binding site of the transition metal Pb in the epiphytic lichens *Hypogymnia physodes* and *Lobaria pulmonaria*. From different pretreatments, which removed isolichenan and other extracellular polysaccharides, lichen substances or the entire extracellular matrix, RICHARDSON et al. (1985) concluded that the cell wall is the binding site for metals.

BRANQUINHO et al. (1997a) found membrane damage and reduced chlorophyll fluorescence in the epiphytic lichens *Ramalina fastigiata* and *Usnea* spec. after incubation with Cu. Studying the influence of Cu and Ni in combination with simulated acid rain on the epiphytic beard lichen *Bryoria fuscescens*, TARHANEN et al. (1999) found that the mycobiont and the photobiont differed in their responses. They concluded, that Cu and Ni damaged more the mycobiont, indicated by a reduction of the ergosterol content, whereas the photobiont seemed more sensitive to acid rain.

Similarly TARHANEN (1998) observed more severe ultrastructural damage in the photobiont than in the mycobiont after treatment with a mixture of Cu, Ni and simulated acid

rain. Damage symptoms in the photobionts included swelling of the thylakoid membranes and cristae in mitochondria.

Effects of Mn on lichens were investigated in only three studies, so far. In *Cladonia rangiferina* BURTON et al. (1981) found a weak K efflux at 100 mMCl_2. Only a Mn concentration as high as 1 M caused significant K efflux.
GOYAL et al. (1982) observed significant K efflux in *Peltigera canina* at 2 – 16 mM Mn, which was lower than at the same concentrations of Cu, but higher than with Ni and Zn.
GARTY et al. (1992) studied the influence of low pH and metals including Mn on the chlorophyll content in the epiphytic lichen *Ramalina lacera* (Syn. *R. duriaei*). All Mn solutions led to chlorophyll degradation, whereby degradation in samples incubated with $MnSO_4$ was more severely than in other sulphate solutions. However, it must be noted that all solutions had been adjusted to a very low pH of 2 and that this condition on its own caused significant chlorophyll degradation.

Mn toxicity in vascular plants

While few studies are available on Mn uptake and toxicity in lichens, there are numerous studies of Mn toxicity in higher plants. Already BORTNER (1935) established that Mn had an influence on tobacco plants. BORTNER (1935) found chlorosis in leaves at low pH and Mn. KITAO et al. (2001) also found leaf chlorosis in *Betula platyphylla* var. *japonica*, the severity of which was correlated with the Mn concentration of the leaves.
Seedlings of *Acer saccharum* treated with a solution containing about 1 mM Mn and higher died almost entirely (MCQUATTIE et al. 2000). Leaf chlorosis could likewise be observed at lower Mn concentration. Furthermore, swelling of cristae in the mitochondria were found in root meristems and discrete electron-dense areas occured in thylakoid membranes.
An extensive overview about Mn toxicity in higher plants was compiled by EL-JAOUAL & COX (1998).

Field observations on Mn toxicity in lichens

In general, epiphytic lichen distribution is controlled by microclimate, structural properties of the substrate and chemical site factors (BARKMAN 1958, HAUCK 2005). In addition, lichen distribution is affected by the continuity of site factors (HILMO 2002, HILMO & SASTAD 2001) and by the effectiveness, by which different species compete for the relevant site factors (WIRTH et al. 1999). Recent field studies that tried to detect effects of chemical site factors are from GAUSLAA (1985, 1995), HOLIEN (1996), GAUSLAA & HOLIEN (1998), JUNG (1998), HAUCK (2000), HESSE (2002) and SCHMULL (2002).

In the Harz Mountains (Germany), an especially high epiphytic lichen diversity was found on Norway spruce (*Picea abies*) affected by pollutant-caused forest dieback. This high diversity included pollution-sensitive lichens in particular. In comparable healthy spruce stands only few pollution-resistent species including the crustose lichen *Lecanora conizaeoides* were found (HAUCK 2000). This phenomenon was also observed by MACHER & STEUBING (1984) in the Bavarian forest. MACHER & STEUBING (1984) as well as JOHN (1986) attributed the occurence of pollution sensitive, epiphytic lichens on damaged trees to increasing light influx and increasing water capacity of the decaying bark of dying and dead trees. This statement was, however, not based on measurements. A totally new explanation for this phenomenon was given by HAUCK & RUNGE (1999, 2002) and HAUCK (2000). They found reduced pollutant concentrations in stemflow of damaged trees due to reduced atmospheric interception on trees with reduced needle mass. The most significant influence exerted S concentration of stemflow. Besides an influence of S, HAUCK et al. (2001, 2002a) found decreasing cover values of epiphytic lichens (including *Hypogymnia physodes*) with increasing Mn content of the substrate or stemflow. Abundance of the toxitolerant *Lecanora conizaeoides*, however, was not affected by Mn concentrations.

It was unlikely that the correlation of the Mn content in bark or stemflow with the cover of lichens such as *Hypogymnia physodes* was coincidental, as numerous chemical parameters were studied and could be ruled out on multiple correlation analysis (HAUCK 2000, HAUCK et al. 2001a, b, 2002, HAUCK & RUNGE 2002).

Negative correlations between Mn concentrations and the abundance of epiphytic lichen species were also found in coniferous forests of western and eastern North America (HAUCK 2003). SCHMULL et al. (2002) observed decreasing cover values of several species with increasing Mn content of stemflow in a mixed forest of *Picea rubens* and *Abies balsamea*. Further, cover of *H. physodes* and *Imshaugia aleurites* decreased with increasing ratio of Mn to Fe concentration in the bark of *Picea rubens* (SCHMULL & HAUCK 2003a).

In *Picea engelmannii – Abies lasiocarpa* forests of Montana negative correlations were found between epiphytic lichen abundance and Mn concentrations in bark of *Picea engelmannii* and *Larix occidentalis* (HAUCK & SPRIBILLE 2005).
The ratios of Mn to Ca, Mg or Fe were partly more closely correlated with epiphytic lichen abundance than the Mn concentration itself (HAUCK & SPRIBILLE 2005). Furthermore the occurence of cyanolichens on conifers in the dripzone of *Populus* trees in Montana and British Columbia was correlated with low bark concentrations of Mn or low Mn/Ca and Mn/Mg ratios, respectively (GOWARD & ARSENAULT 2000, HAUCK & SPRIBILLE 2002).

Mn concentration in conifer bark primarily depends on Mn concentrations in the soil (HAUCK et al. 2002a), as Mn is readily taken up by the tree roots and transfered to the bark through the xylem and xylem parenchyma (LÖVESTAM et al. 1990, SLOOF & WOLTERBEEK 1993). Mn concentrations in stemflow are primarily controlled by leaching rates from bark and foliage (LEVIA & HERWITZ 2000), whereas interception of Mn from the atmosphere is of subordinate significance (HAUCK 2003). Thus Mn is primarily a natural site factor for epiphytic lichens, the significance of which may be increased due to anthropogenic soil acidification, as Mn availability increases with decreasing pH (NORVELL 1988).

Objective of the present study

The aim of the present study was to test the hypothesis that decreasing epiphytic lichen abundance found with increasing Mn supply from bark or stemflow in coniferous forests of Europe and North America was due to toxic effects of excess concentrations of Mn.

Studies were carried out with selected species from coniferous forests from Europe and North America. Most experiments were conducted with the foliose lichen *Hypogymnia physodes*, which appeared to be Mn sensitive from field data. This species is widespread in coniferous forest of the northern hemisphere and its soredia can easily be cultured so that the species is well suited as a model organism. Further investigations were carried out in *Alectoria sarmentosa* and *Bryoria fuscescens*.

Lecanora conizaeoides was studied as an apparently Mn tolerant lichen, for which no correlation between its abundance and Mn concentrations in bark or stemflow was found. *L. conizaeoides* was already known for its extreme tolerance to SO_2 (HAUCK et al. 2001a).

Mn sensitivity of cyanolichens was studied in the homoiomerous, bipartite lichen *Leptogium saturninum*, in the heteromerous, bipartite lichen *Nephroma helveticum* and in the heteromerous, tripartite lichen *Lobaria pulmonaria*.

The study is subdivided as follows:

Chapter 2 discusses the influence of Mn on soredia in *Hypogymnia physodes* and interaction with Ca and Mg. The chapter is based on culture experiments with soredia at 7 µM and 7 mM Mn. Soredia growth and chlorophyll concentrations were studied. Element distribution in the soredia was determined with X-ray microanalysis and simultaneously structural changes were studied with TEM and SEM.

Chapter 3 focusses on element microdistribution in soredia of *Hypogymnia physodes* cultivated at 500 µM for varying cultivation periods. Element content was studied with X-ray microanalysis combined with TEM.

Chapter 4 examines ultrastructural changes in soredia of *H. physodes*, cultured at different Mn concentrations (7 µM, 500 µM and 7 mM Mn).

Chapter 5 studies extracellular adsorption and intracellular uptake in thalli of *Hypogymnia physodes* and *Lecanora conizaeoides*. In addition, conversion of Mn^{2+} to insoluble Mn^{3+} and Mn^{4+} in the apoplast was studied.

Chapter 6 deals with the element distribution in thalli of Mn supplemented and unsupplemented *H. physodes* and *L. conizaeoides* on a subcellular level. Furthermore, thallus structures were observed with a TEM and SEM.

Chapter 7 is about the influence of Mn on the chlorophyll concentrations in thalli of *H. physodes* and *L. conizaeoides* incubated with Mn alone or in combination with Ca, Mg or Fe. Further, the effect of Mn on ATP concentrations was studied in thalli of *H. physodes* to test the hypothesis that high Mn concentration cause intracellular P deficiency.

Chapter 8 studies Mn adsorption and uptake in the beard lichens *Alectoria sarmentosa* and *Bryoria fuscescens*. Simultaneously, effects of Mn on membrane integrity was investigated.

Chapter 9 focusses on Mn adsorption and uptake in the cyanolichens *Leptogium saturninum*, *Lobaria pulmonaria* and *Nephroma helveticum*.

Chapter 10 studies effects of Mn on chlorophyll parameters in the chlorolichen *Hypogymnia physodes* and the cyanolichens *Leptogium saturninum*, *Lobaria pulmonaria* and *Nephroma helveticum*. Parameters studied were Φ_2, NPQ, qN and qP.

Chapter 11 compares effects of excess Mn on the photobionts of *Hypogymnia physodes*, *Lecanora conizaeoides*, *Leptogium saturninum*, *Nephroma helveticum* and *Lobaria pulmonaria* by means of fluorescence microscopy.

Chapter 12 discusses experimental data of Mn toxicity against the background of field data in summary.

Chapter 2

Effects of manganese on the viability of vegetative diaspores of the epiphytic lichen *Hypogymnia physodes*

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Abstract

Soredia of the lichen *Hypogymnia physodes* cultivated with Bold's basal medium on agar plates for 8 days exhibited decreasing growth rates along with increasing Mn concentrations above 3 mM. Ca and Mg, added separately or in combination, alleviated Mn toxicity. The chlorophyll a and b content of the soredia was reduced under the influence of Mn and was positively correlated with the rate of grown soredia. *Trebouxia* cells of the soredia grown with excess Mn were smaller than control cells, had reduced chloroplasts and were partly collapsed; fungal hyphae were shortened and strongly swollen. Disintegrated cell walls occurred both in algal and fungal cells. Excess Mn was sequestered in extracellular encrustations together with phosphate as corresponding anion. Intracellularly, Mn was accumulated in polyphosphate granules both in algal and fungal cells. Mn uptake was correlated with significant loss of Na, Mg and Ca, particularly from the mycobiont. Fungal cell walls also lost significant amounts of P. The same damage symptoms occurred in cells of soredia both grown or not, but the former had a higher share of intact cells. Damaged cells of both types of soredia had equally increased Mn concentrations, whereas total Mn content was higher in not grown soredia than in grown ones due to the greater amount of damaged cells in the former. The Si/Mn ratio in cell walls of intact *Trebouxia* cells was significantly higher than in collapsed cells. The experimental evidence of Mn sensitivity of *H. physodes* soredia corresponds to studies of epiphyte vegetation in montane spruce forests of northern Germany that revealed decreasing cover values of *H. physodes* with increasing Mn content of the substrate.

1. Introduction

HAUCK (2000) and HAUCK et al. (2001b) established a negative correlation between the cover of the foliose lichen *Hypogymnia physodes* and the Mn content of spruce bark in several stands of *Picea abies* in the Harz Mountains, northern Germany. The total number of lichen species per tree also decreased with increasing Mn content of the bark. However, cover of the crustose lichen *Lecanora conizaeoides*, which is known to be particularly tolerant to chemical agents (WIRTH 1995), was not correlated with the Mn content of the substrate. The Mn content of the spruce bark in the investigated stands ranged from 0.3 - 11 mmol kg^{-1} d. wt. and correlated with the Mn content of soil (HAUCK 2000). From the soil Mn is taken up by the root system and deposited in the bark after xylem transport (LÖVESTAM et al. 1990).

Although the sensitivity of lichens to heavy metals has often been studied, information on effects of Mn on lichens is sparse (NIEBOER et al. 1978, NASH 1989). WILSON & JONES (1984) established that the crustose lichen *Pertusaria corallina* immobilizes Mn as an oxalate salt at the lichen/rock interface, when growing on Mn-rich ores. BURTON et al. (1981) and GOYAL & SEAWARD (1982) measured the K levels of *Cladonia rangiferina* and *Peltigera canina*, respectively, subsequent to short-term exposure to Mn solutions. Significant K losses occurred in the former species, which has a green algal photobiont only, at Mn concentrations as high as 1 M. In the latter species, which has a cyanobacterial photobiont, 2 - 16 mM of Mn were sufficient to reduce the total K content of the thallus by up to 70 %. However, no study to date has revealed that Mn could be a limiting factor for lichen performance in the field. Therefore, we studied the sensitivity of some species to Mn in order to test whether the negative correlations between lichen occurrence and Mn content of bark that were found in the Harz Mountains could have been due to a toxic effect of Mn. In the present paper, we report on culture experiments with soredia of *Hypogymnia physodes*, investigating the effects of Mn alone and in combinations with Ca and Mg. Our aim was to test the hypothesis that, firstly, the growth of *H. physodes* soredia decreases with increasing Mn supply in ambient concentration ranges and that, secondly, growth does depend not solely on the Mn content itself, but decreases with increasing Mn/Ca and Mn/Mg ratios of the substrate. In addition to the studies on soredia growth, the chlorophyll content of differently affected soredia was measured and electron-microscopy studies were carried out in

order to detect possible mechanisms of Mn toxicity and to clarify whether both the mycobiont and the photobiont are damaged by Mn.

2. Materials and methods

2.1. Soredia growth

Growth of soredia of *Hypogymnia physodes* (L.) Nyl. was studied according to MARGOT (1973) and MARTI (1985). *H. physodes* soredia consist of a few photobiont cells [*Trebouxia jamesii* (Hildreth and Ahmadjian) Gärtner; AHMADJIAN 1993] surrounded by fungal hyphae. The lichen material was collected a few days before usage and stored at c. 5 °C in the dark. Growth studies were carried out in Petri dishes with Bold's basal medium, a mineral agar medium prepared according to AHMADJIAN (1993). The medium contains 2.9 mM $NaNO_3$, 1.3 mM KH_2PO_4, 430 µM K_2HPO_4, 300 µM $MgSO_4$, 220 µM $CaCl_2$, 430 µM NaCl, 185 µM H_3BO_4, 18 µM $FeSO_4$, 31 µM $ZnSO_4$, 7 µM $MnCl_2$, 5 µM MoO_3, 6 µM $CuSO_4$, 2 µM $Co(NO_3)_3$, 130 µM Na_2EDTA, and 550 µM KOH. In the first experiment, $MnCl_2$ was added to the medium in concentrations of 1, 3, 7, and 10 mM; these Mn concentrations correspond to concentrations found in spruce bark in the Harz Mountains. Media were adjusted to pH 3.1 with HCl, which corresponds to the pH found in bark and stemflow of Norway spruce (HAUCK 2000). Controls without additional Mn (i.e. a basal concentration of 7 µM $MnCl_2$) were run at pH 3.1 and 6.1. The latter is within the optimum range for soredia growth of *H. physodes*, whereas MARTI (1985) found a slightly reduced growth rate at pH 3. In a second experiment, 3 mM $MnCl_2$ at pH 3.1 were applied together with either $MgCl_2$ or $CaCl_2$ or with a combination of both (0.5, 3 mM Mg; 1, 3 mM Ca; 0.5 mM Mg + 1 mM Ca). Controls for 3 mM Mg and 3 mM Ca, respectively, were run without additional Mn (pH 3.1). Concentrations given for Mn, Ca and Mg are in addition to the amounts supplied by Bold's basal medium, resulting in total Mg concentrations of 0.8 and 3.3 mM and in total Ca concentrations of 1.2 and 3.2 mM. In the following, we refer to the culture media as Mn 0, 1, 3, 7, 10, Mn 3 + Mg 0.5, Mn 3 + Mg 3, Mn 3 + Ca 1, Mn 3 + Ca 3, Mn 3 + Mg 0.5 + Ca 1, Ca 3, and Mg 3 mM, specifying the additional concentrations.

About 500 soredia were put on each Petri dish, which were incubated for 8 days at 80 % rh, a day temperature (for 13 hours daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1} and a night temperature of 10 °C. The first assay without Mg and Ca was carried out in five replicates, the second one in four replicates. After incubation small, whitish, not grown soredia and green, larger soredia, where propagation of algal cells took place, were separately counted (MARGOT 1973). Viability of soredia of *H. physodes* varies widely even under optimal growth conditions depending on the season. Therefore, the tests were run in winter and early spring when viability is highest (FIECHTER & HONEGGER 1988). The rates of grown soredia are given as percent of the respective controls.

2.2. Chlorophyll content of soredia

The chlorophyll content of the soredia of the first growth assay (without Mg and Ca) was measured in order to test the hypothesis that an Mn-induced inhibition of chlorophyll formation could be the cause for a reduction in soredia growth with Mn incubation. This hypothesis was based on the light-microscopy observation that chloroplasts in not grown soredia had a lighter green color than in grown ones. Two replicates of about 150 - 200 soredia of each Petri dish were transferred into testtubes and incubated with 3 ml N,N-dimethyl formamide (DMF) for 24 h (MORAN 1982). In a preliminary test, DMF was found to be more effective in pigment extraction from *H. physodes* than dimethyl sulfoxide (DMSO; RONEN & GALUN 1984). The absorbance of the extracts was determined with a Shimadzu photometer (UV 120/02) at λ = 603, 625, 647 and 664 nm. The content of chlorophylls a and b (Chl a, Chl b) was calculated according to the following equations, which are particularly suitable for low concentration ranges (Moran 1982): Chl a [ppm] = 12.81 A_{664} - 2.16 A_{647} - 1.44 A_{625} - 4.91 A_{603}; Chl b [ppm] = -4.93 A_{664} - 26.01 A_{647} + 3.74 A_{625} - 15.55 A_{603}. Pigment concentrations were related to 100 soredia. Arithmetic means of the two replicates of each Petri dish were calculated before statistical analysis.

2.3. Preparation for transmission and scanning electron microscopy

H. physodes soredia were incubated with (7 mM) and without (7 µM) excess $MnCl_2$ for 8 days on agar plates as described above. Grown and not grown soredia both of the control and the Mn-stressed variant were separated by cutting the agar into blocks of 2 x 2 mm under the dissecting microscope. The agar blocks were rapidly frozen in a 2 : 1 mixture of propane and isopentane cooled with liquid nitrogen to –196 °C, freeze-dried at –45 °C for 3 days and stored at 20 °C in a desiccator over silica gel.

2.4. X-ray microanalysis in the transmission electron microscope

Freeze-dried agar blocks with soredia were infiltrated with ether in a vacuum-pressure chamber and embedded in styrene-methacrylate (FRITZ 1989). Blocks were cut with dry glass knives with an ultramicrotome in 0.5 – 1.0 µM thick sections. The sections were mounted on adhesive-coated 100-mesh hexagonal grids (FRITZ 1991), coated with carbon and stored over silica gel. The sections were analyzed in a Philips EM 420 with the energy dispersive system EDAX DX-4 (EDAX Inc., Mahwah, NJ 07430, U.S.A.). The accelerating voltage was 120 kV, the take-off angle 25° and the counting time 60 live seconds. Quantitative data (mmol dm^{-3} element content) were obtained as described by FRITZ & JENTSCHKE (1994), taking into account the calibration coefficients (Cliff-Lorimer factors) of the elements relative to K.

2.5. X-ray microanalysis in the scanning electron microscope

Freeze-dried agar blocks with soredia were mounted on specimen holders, gold-coated and examined with a Philips SEM 515 operating at 20 kV. Identically treated but carbon-coated samples were employed for X-ray microanalysis with an energy dispersive EDAX PV 9100 system. The accelerating voltage was 20 kV, the take-of angle 36 ° and the counting time 60 live seconds. The data were presentend as ratios of X-ray counts in the element peak to X-ray counts in the underlying background.

2.6. Statistics

Arithmetic means ± SE are given in all tables and figures. After testing data for normal distribution with the Shapiro-Wilk test, Student's t-test was employed for determining significant differences between samples. Pearson's product-moment was calculated for correlation analysis of binormally distributed data. A bivariate analysis of variance (ANOVA) was carried out in order to test whether the Mn supply with the culture medium or the vitality of the soredium was decisive for the Mn content of algal and fungal cell compartments (Table 2-5). The ANOVA quantifies in percent to what extent variation of the Mn content can be attributed to each of the independent variables. Statistical significance was tested by calculating F values (BORTZ 1999). Statistical analyses were computed with SAS 6.04 software (GOGOLOK et al. 1992).

3. Results

3.1. Soredia growth

The rate of grown *H. physodes* soredia decreased significantly with increasing Mn content of the culture medium (Table 2-1). Soredia growth of the controls at pH 6.1 did not differ from that at pH 3.1. Addition of 3 mM Ca or Mg reduced the soredia growth compared to the control, but mitigated the inhibiting effect of Mn (Fig. 2-1). A combination of 0.5 mM Mg and 1 mM Ca completely compensated for the growth inhibition at 3 mM Mn.

Table 2-1. Viability and chlorophyll content of *Hypogymnia physodes* soredia at different Mn concentrations of the culture medium at pH 3.1 and at pH 6.1 without Mn addition.

Mn [mM] in culture medium	pH	Grown soredia [%][a]		Chl a [µg/100 sor.][b]		Chl b [µg/100 sor.][b]	
0	6.1	102 ± 3	a	0.44 ± 0.07	a	0.30 ± 0.08	a
0	3.1	100 ± 6	a	0.40 ± 0.09	ab	0.22 ± 0.07	ab
1	3.1	91.5 ± 5.3	a	0.34 ± 0.10	abc	0.18 ± 0.04	ab
3	3.1	68.9 ± 5.6	b	0.27 ± 0.07	abc	0.16 ± 0.04	ab
7	3.1	25.2 ± 5.1	c	0.21 ± 0.06	bc	0.13 ± 0.02	ab
10	3.1	4.59 ± 1.02	d	0.13 ± 0.04	c	0.10 ± 0.02	b

[a] Rate of grown soredia specified as percentages of the control at pH 3.1. Statistics: t-test, $P \leq 0.05$, df = 4; different letters indicate significant difference within column.
[b] Mean chlorophyll concentrations. Statistics: cf. [a].

3.2. Chlorophyll content of soredia

Both the Chl a and the Chl b content of the soredia decreased with increasing Mn content in the culture medium. The content of Chl a in the 10 mM Mn assay was reduced to 32 % of the control at pH 3.1 and to 29 % of the control at pH 6.1, respectively (Table 2-1). The Chl b content at 10 mM Mn amounted to 45 % of the pH 3.1 control and to 33 % of the pH 6.1 control (Table 2-1). The rate of grown soredia increased with their content of Chl a and b (Figs. 2-2, 2-3).

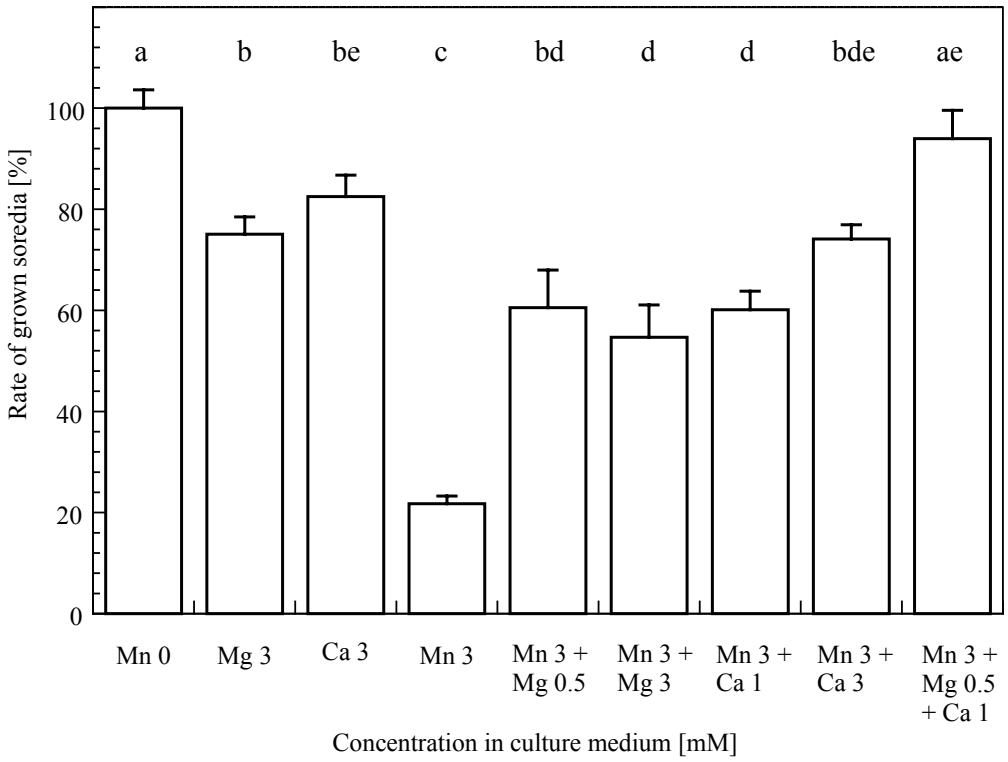

Figure 2-1. Effects of Mg and Ca on Mn-induced inhibition of soredia germination in *Hypogymnia physodes* at pH 3.1. Germination rates are specified as percentages of the control. Significant differences are indicated by different letters above the columns (t-test, $P \leq 0.05$, df = 3). Vertical bars indicate ± SE.

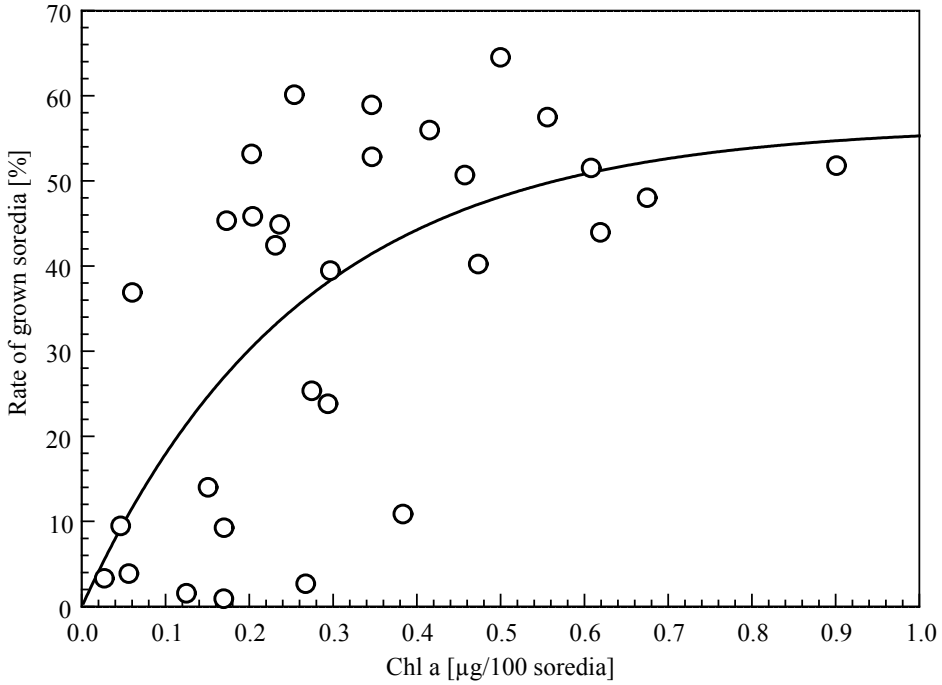

Figure 2-2. Germination of *Hypogymnia physodes* soredia vs Chl a content ($r_{nl} = 0.64$, $P \leq 0.001$, df = 27; regression model: $y = a + b [1 - e^{cx}]$).

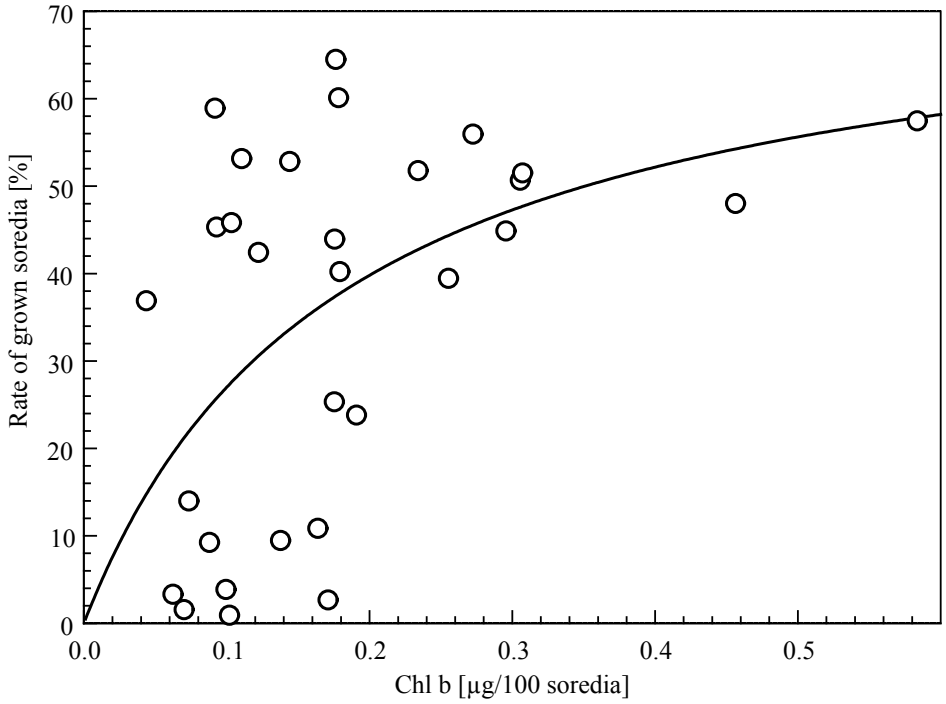

Figure 2-3. Germination of *Hypogymnia physodes* soredia vs Chl b content ($r_{nl} = 0.50$, $P \leq 0.01$, df = 28; regression model: y = [ax]/[x + b]).

3.3. SEM studies

Grown soredia of the control had a dense network of thin and long fungal hyphae with a diameter of less than 1 µm. Photobiont cells were covered by this fungal network and were up to 20 µm in diameter (Fig. 2-4A). Not grown soredia of the 7 mM Mn assay were characterized by smaller *Trebouxia* cells as well as by strongly swollen and shortened hyphae of the mycobiont (Fig. 2-4B). Many algal cells were collapsed. Soredia were frequently covered with encrustations (Fig. 2-4C), which were shown to consist primarily of Mn and P by X-ray microanalysis (Table 2-2). Mycobiont and photobiont cells of grown soredia, which had been cultivated with 7 mM Mn, contained considerably enhanced levels of Mn and P (Table 2-2). Comparing the element content of grown soredia and not grown ones within the 7 mM Mn assay revealed that the concentration of Mn and P was considerably higher in the ones not grown. Photobiont cells in not grown soredia of the control were smaller than those in grown soredia, but, in contrast to the soredia in the 7 mM Mn assay, the photobiont was surrounded by a fine network of thin fungal hyphae (Fig. 2-4D).

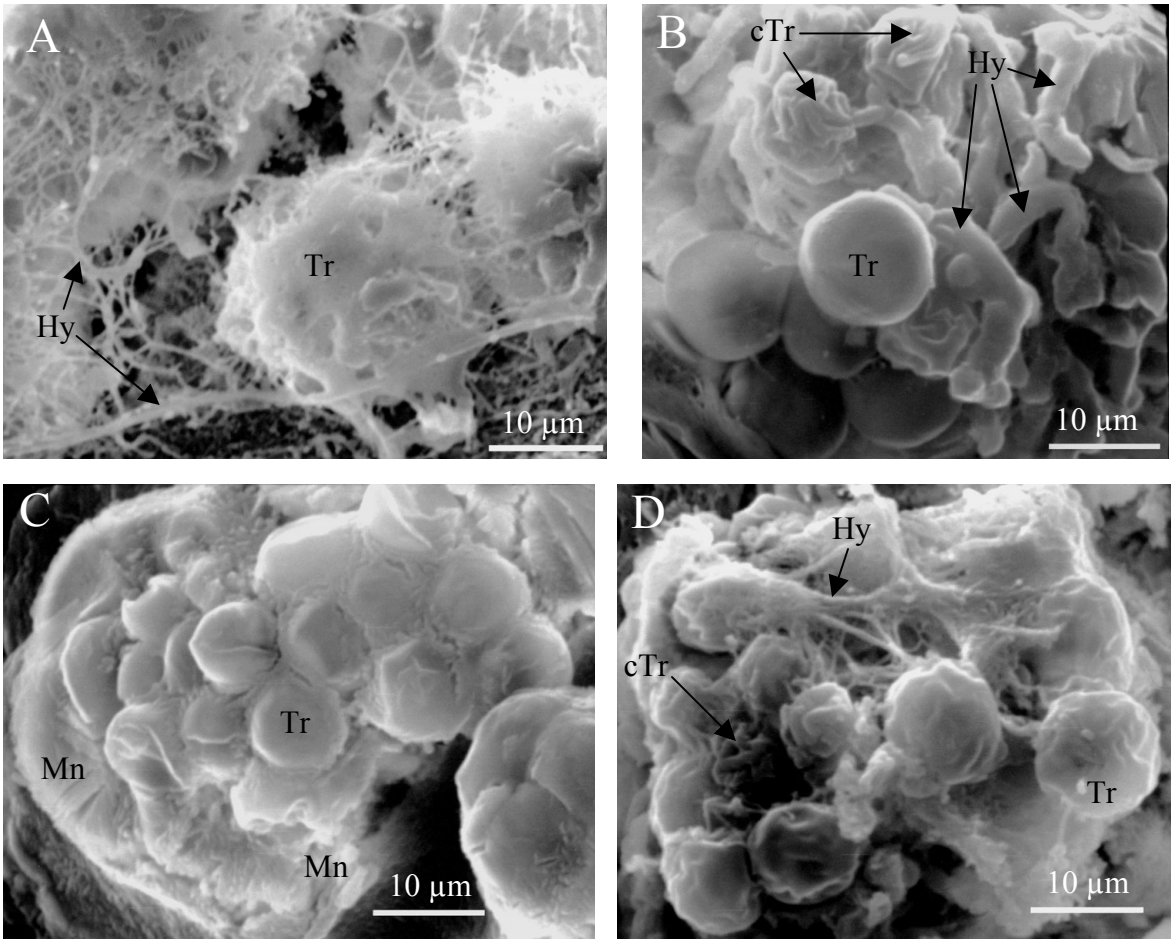

Figure 2-4. SEM micrographs of soredia of *Hypogymnia physodes*. A. Germinated soredium from the control with a network of densely branched thin hyphae (Hy) covering cells of the photobiont *Trebouxia* (Tr). B. Ungerminated soredium subsequent to incubation with 7 mM Mn. Hyphae (Hy) are strongly swollen. Cells of *Trebouxia* (Tr) are smaller than in germinated soredia of the control and partly collapsed (cTr). C. Ungerminated soredium of 7 mM Mn assay with encrustations of immobilized Mn (Mn) and small *Trebouxia* cells (Tr). D. Ungerminated soredium of the control. Photobiont cells (Tr) are particularly smaller than in germinated soredia or even collapsed (cTr) as in soredia of the 7 mM Mn assay, but fungal hyphae (Hy) are normally developed.

Table 2-2. X-ray microanalysis (SEM) of *Hypogymnia physodes* soredia cultivated with either 7 µM or 7 mM Mn (peak/background ratios).

	Variant[a]	Na*K*	Mg*K*	Si*K*	P*K*	S*K*	Cl*K*	K*K*	Ca*K*	Mn*K*	Fe*K*	*n*[b]
Soredia	Mn0+	0.90	0.40	0.14	1.24	1.00	0.51	2.83	0.21	0.00	0.09	5
"	Mn0-	0.67	0.38	0.25	1.59	1.26	0.62	4.01	0.23	0.00	0.00	3
"	Mn7+	0.70	0.25	0.28	1.19	0.93	3.16	1.86	0.13	2.45	0.12	2
Soredia, fungal hyphae	Mn7-	0.29	0.14	0.20	10.5	0.34	1.33	0.71	0.19	36.0	0.12	3
Soredia, algal cells	Mn7-	0.12	0.19	0.22	5.74	0.62	1.71	1.93	0.16	18.0	0.35	3
Encrustations	Mn0+	0.40	0.39	0.15	1.38	0.83	0.50	3.27	0.19	0.00	0.00	4
"	Mn0-	0.74	0.41	0.19	1.68	1.01	0.72	3.43	0.19	0.00	0.00	2
"	Mn7+	0.22	0.27	0.17	1.51	0.98	1.35	2.57	0.09	1.87	0.16	4
"	Mn7-	0.00	0.00	0.20	9.67	0.14	1.33	0.61	0.25	26.6	0.22	3
Agar medium	Mn7	0.08	0.13	0.28	0.57	1.12	5.77	1.16	0.28	4.39	0.07	3

[a] Variants: Mn0+ — Grown soredia from the control (7 µM Mn). Mn0- — Not grown soredia from the control. Mn7+ — Grown soredia cultivated with 7 mM Mn. Mn7- — Not grown soredia cultivated with 7 mM Mn.
[b] *n* — Number of samples.

3.4. TEM studies

Trebouxia cells of the control were almost completely filled with the chloroplast. Dark bodies scattered over the cells were identified as polyphosphate granules by X-ray microanalysis (Fig. 2-5E, Table 2-3). Polyphosphate granules were also found in fungal hyphae (Fig. 2-5C). In soredia supplied with excess Mn, thylakoids were reduced or completely lacking. Both algal and fungal cells contained numerous dark granules (Fig. 2-5A), which mainly consisted of Mn and P (Table 2-3). Mn-containing polyphosphate granules were more sharpely limited and had a higher electron density than polyphosphate bodies of the control. Collapsed *Trebouxia* cells with disintegrated cell walls frequently occurred both in soredia grown or not (Fig. 2-5D). Furthermore, fungal cells containing many Mn/P granules sometimes had incomplete cell walls. Dark ellipsoid encrustations rich in Mn and P were found forming the outline of an algal cell (Fig. 2-5B). Element content in the interior of these structures differed from that of the agar medium, identifying them as destroyed *Trebouxia* cells. X-ray microanalysis of such encrustations yielded an Mn concentration of 8135 mmol dm^{-3} and a P concentration of 5265 mmol dm^{-3} (sum spectrum of five measurements).

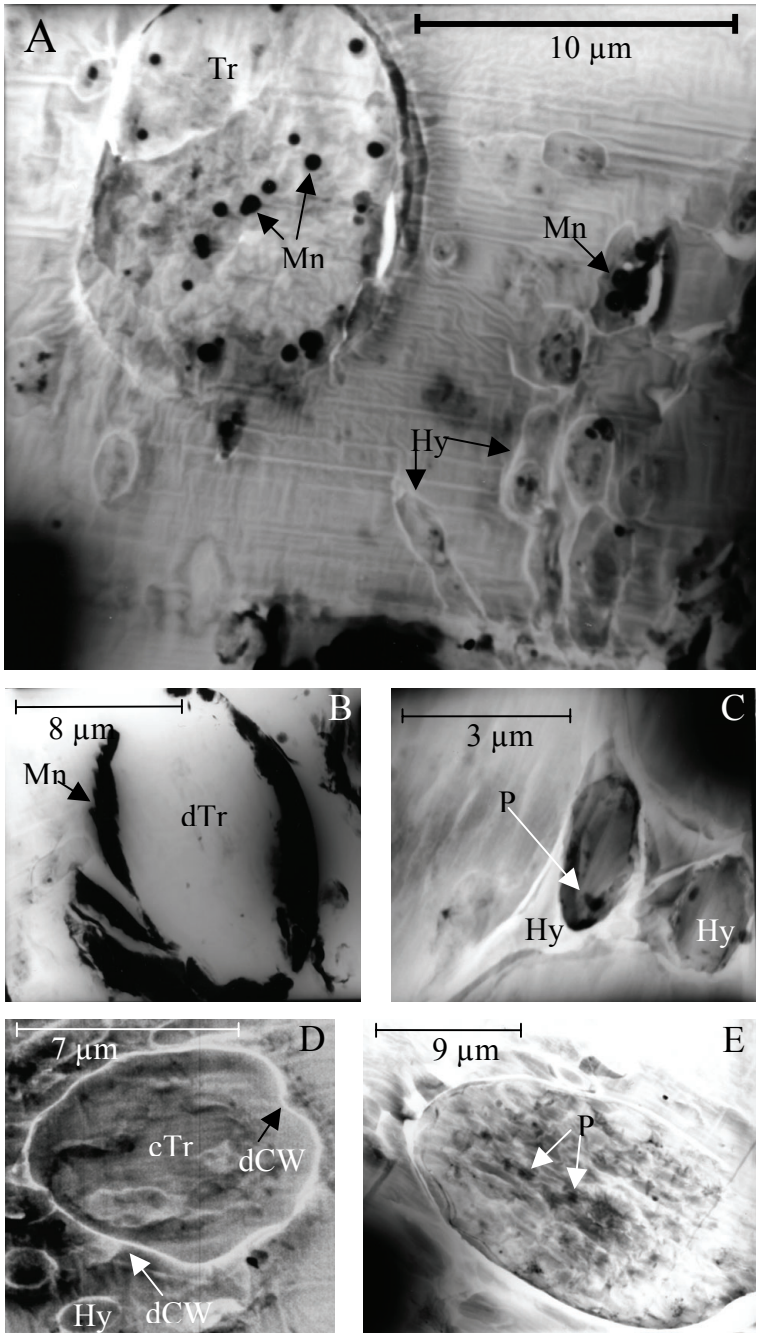

Figure 2-5. TEM micrographs of soredia of *Hypogymnia physodes*. A. Ungerminated soredium cultivated with 7 mM Mn. Photobiont (Tr) and mycobiont cells (Hy) contain numerous polyphosphate granules rich in Mn (Mn). B. Disintegrated *Trebouxia* (dTr) cell with thick encrustations of Mn and P (Mn) in soredium cultivated 7 mM Mn. C. Fungal hyphae (Hy) in germinated soredium of the control containing polyphosphate granules (P). D. Collapsed *Trebouxia* cells (cTr) with partly disintegrated cell walls (dCW) surrounded by fungal hyphae (Hy) in soredium cultivated with 7 mM Mn. E. Photobiont cell in germinated soredium of the control with polyphosphate granules (P).

Table 2-3. X-microanalysis (TEM) of mycobiont cells in *Hypogymnia physodes* soredia incubated with (Mn 7 mM) and without (Mn 0 mM) excess Mn. Concentrations in mmol dm^{-3}.

	Polyphosphate granules		Cell lumina		Cell walls	
	Mn 0 (n = 4)[a]	Mn 7 (n = 6)	Mn 0 (n = 4)	Mn 7 (n = 3)	Mn 0 (n = 4)	Mn 7 (n = 6)
Mn	0.00 ± 0.00[b]	928 ± 253*	0.00 ± 0.00	52.5 ± 11.0*	0.00 ± 0.00	37.6 ± 3.8***
K	963 ± 220	341 ± 100	77.2 ± 8.5	82.3 ± 13.7	251 ± 36	153 ± 27
Na	743 ± 231	66.2 ± 11.3**	93.5 ± 29.6	31.0 ± 3.1	235 ± 61	73.6 ± 10.5*
Ca	188 ± 111	15.7 ± 5.6	16.8 ± 3.6	2.95 ± 2.95*	11.0 ± 2.2	4.20 ± 1.77*
Mg	798 ± 88	127 ± 32**	28.8 ± 4.2	21.8 ± 4.5	45.7 ± 13.5	1.67 ± 1.67**
Fe	0.00 ± 0.00	3.20 ± 2.69	3.20 ± 1.14	2.29 ± 1.33	0.75 ± 0.75	1.94 ± 0.41
Si	34.8 ± 16.4	49.5 ± 25.8	47.1 ± 10.0	46.6 ± 17.1	71.4 ± 22.8	88.1 ± 33.9
P	3680 ± 656	2140 ± 803	125 ± 8	134 ± 7	183 ± 55	36.9 ± 6.9*
S	96.8 ± 39.9	80.3 ± 18.5	46.0 ± 9.6	59.0 ± 5.7	54.2 ± 8.8	38.0 ± 3.6
Cl	65.2 ± 17.1	54.4 ± 13.2	45.0 ± 14.0	45.0 ± 12.5	93.7 ± 14.2	109 ± 10

[a] n — Number of sum spectra (each computed of 5 - 8 single spectra).
[b] Arithmetic means ± standard error. Asterisks indicate significant difference between Mn 0 and Mn 7 assays.
Levels of significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (t-test).

Table 2-4. X-microanalysis (TEM) of photobiont cells in *Hypogymnia physodes* soredia incubated with (Mn 7 mM) and without (Mn 0 mM) excess Mn. Concentrations in mmol dm^{-3}.

	Polyphosphate granules		Cell lumina		Cell walls	
	Mn 0 (n = 5)[a]	Mn 7 (n = 7)	Mn 0 (n = 7)	Mn 7 (n = 5)	Mn 0 (n = 5)	Mn 7 (n = 9)
Mn	0.00 ± 0.00[b]	1140 ± 138***	0.00 ± 0.00	66.2 ± 36.3*	0.00 ± 0.00	58.0 ± 22.1*
K	484 ± 86	512 ± 58	153 ± 24	191 ± 1	143 ± 26	243 ± 22*
Na	200 ± 84	112 ± 36	42.5 ± 27.5	105 ± 27	126 ± 45	195 ± 44
Ca	6.07 ± 5.28	21.1 ± 11.1	0.42 ± 0.42	1.91 ± 0.84	5.66 ± 1.14	6.39 ± 1.34
Mg	356 ± 113	105 ± 10*	31.2 ± 10.4	55.3 ± 17.1	13.8 ± 2.4	27.3 ± 9.6
Fe	1.26 ± 0.85	1.54 ± 1.54	1.64 ± 0.42	2.85 ± 0.31*	1.35 ± 0.61	2.46 ± 0.51
Si	264 ± 228	77.7 ± 12.9	47.9 ± 12.7	51.8 ± 11.9	142 ± 38	119 ± 32
P	1190 ± 317	2920 ± 479*	99.9 ± 25.4	336 ± 174	75.8 ± 17.4	165 ± 45
S	107 ± 12	90.9 ± 19.4	96.1 ± 14.8	124 ± 30	43.6 ± 15.1	69.4 ± 14.0
Cl	37.2 ± 4.7	74.9 ± 19.4	33.2 ± 6.2	89.5 ± 24.2*	74.2 ± 30.1	195 ± 35*

[a] n — Number of sum spectra (each computed of 4 - 9 single spectra).
[b] Arithmetic means ± standard error. Asterisks indicate significant difference between Mn 0 and Mn 7 assays.
Levels of significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (t-test).

Table 2-5. Effects of culture medium (0/7 mM Mn) and soredia viability (grown/not grown) on the Mn concentration in cell compartments of *Hypogymnia physodes* soredia.

	n[b]	Total		Culture medium		Growth		Culture medium × growth	
		Var. [%]	F value	Var. [%]	F value	Var. [%]	F value	Var. [%]	F value
MPP[a]	10	57	2.61	54	7.52*	0	0.03	2	0.28
MLU	7	97	37.41**[c]	91	104.51**	0	0.28	6	7.44
MCW	10	89	16.39**	84	46.58**	4	2.46	0	0.12
PPP	12	83	12.62**	76	34.63***	7	3.20	0	0.02
PLU	12	38	1.66	27	3.56	7	0.93	0	0.50
PCW	14	24	1.03	16	2.11	7	0.97	0	0.01

[a] Cell compartments analyzed with X-ray microanalysis (mycobiont/photobiont): MPP/PPP — Polyphosphate granules, MLU/PLU — Cell lumina, MCW/PCW — Cell walls.
[b] *n* — Number of sum spectra.
[c] Levels of significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (ANOVA).

Fungal and algal cells of soredia cultivated with excess Mn contained significantly enhanced concentrations of Mn in cell walls, cell lumina and polyphosphate granules (Table 2-3). In the mycobiont cells, Mn accumulation did not alter the concentration of P in the polyphosphate granules, but Mn replaced cations such as Na, Mg, K and Ca. In *Trebouxia*, accumulation of Mn in polyphosphate granules led to a significant increase in their P content (Table 2-4). The content of Mn was significantly correlated with that of P in the polyphosphate granules of the algal cells ($r = 0.76$, $P \leq 0.01$, $df = 11$), but not in the fungal hyphae. Mn accumulation in the algal polyphosphate granules also resulted in a significant decrease of the Mg content in the granules (Table 2-3). Elevated Mn concentrations in the cell lumina were correlated with reduced Ca concentrations in the mycobiont and an increase in Cl content in the photobiont. The fungal cell walls of soredia supplied with excess Mn contained less Na, Ca, Mg and P than those of the controls (Table 2-3). In *Trebouxia*, cell wall concentrations of K and Cl were higher in the assay with excess Mn than in the controls (Table 2-4). Cell walls in collapsed *Trebouxia* cells of the 7 mM Mn assay exhibited a significantly lower Si/Mn ratio (1.31 ± 0.47) than in intact cells of the same assay (3.99 ± 0.47; t-test, $P \leq 0.05$, $df = 2$). This difference between collapsed and intact cells occurred both in soredia that were grown or not. In general, element content of a single cell did not depend on whether the re-

spective soredium had been grown, as shown for Mn in Table 2-5, but on individual cell vitality.

4. Discussion

The study clearly revealed a negative effect of the Mn concentration of the culture medium on soredia growth. Growth experiments with soredia of *H. physodes* as introduced by MARGOT (1973) and MARTI (1985) are well suited for tests of the toxicity of chemical agents on the early stages of its development. Since *H. physodes* very rarely forms apothecia, the occurrence of *H. physodes* in the field is closely linked to the viability of its soredia. ARMSTRONG (1987) established that most soredia of *H. physodes* were deposited within 2 cm of the thallus. Thus, a close correlation between soredia viability and cover values, which can easily be determined in the field, can be expected. A disadvantage of the soredia growth tests is their limited reproducibility. Rates of grown soredia are only comparable within one and the same experiment. This is, first of all, due to the considerable seasonal variation in the ability of *H. physodes* of starting to grow (FIECHTER & HONEGGER 1988). Equal rates of grown soredia at pH 3.1 and 6.1 without Mn addition led to the conclusion that the low pH of spruce bark in the Harz Mountains has no significant effect on soredia viability (and, thus, on cover values) of *H. physodes*. This is not in accordance with results of MARTI (1985), who reported slightly reduced growth at pH 3; however, he did not test his results for statistical significance. The data shown in Fig. 2-1 rule out the possibility that the decrease of soredia growth was not due to Mn, but to chloride. If chloride was decisive, Mn supplied in combination with Ca and/or Mg should have led to even lower rates of soredia growth than in the tests with Mn alone, as the chloride concentration was up to twice as high in these variants. Furthermore, it can be taken from Fig. 2-1 that soredia growth is much less with 3 mM Mn than with 3 mM Ca or 3 mM Mg. This rules out the possibility that the decrease of soredia growth with increasing Mn supply is just a response to the ionic status of the substrate in general.

It is evident from our chlorophyll measurements that a correlation exists between the chlorophyll content of the soredia and their viability. This is in accordance with the observation made with the TEM that thylakoid membranes were disintegrated in Mn-

affected soredia. In seedlings of *Acer saccharum* supplied with excess Mn, MCQUATTIE & SCHIER (2000) established electron-dense regions associated with thylakoid membranes. SABNIS et al. (1969) found Pb to be primarily accumulated in stroma thylakoids when investigating pea chloroplasts. They attributed the affinity of Pb and other heavy metals for the stroma thylakoids to the low electrode potential of ferredoxins. Membrane-bound ferredoxin is restricted to the PS I within the chloroplasts, which is primarily located in the stroma thylakoids in vascular plants (MALKIN & BEARDEN 1978). A more significantly reduced Chl a than Chl b content in Mn-affected soredia suggests that the same mechanism occurs in *H. physodes* as well, since the Chl a/Chl b ratio is higher in PS I than in PS II.

Our SEM studies revealed that not only the photobiont, but also the mycobiont was damaged by Mn, whereas no morphological alterations could be detected in fungal hyphae of the control. X-ray microanalysis combined with SEM showed enhanced Mn concentrations in all soredia of the assay with excess Mn; however, the Mn content was less elevated in soredia that had been grown than those that had not. With TEM, elevated Mn concentrations were found in all components of both algal and fungal cells. The ubiquitous intra- and extracellular occurrence of Mn can be explained by its capability of binding with many types of ligands due to its physico-chemical properties as a borderline ion with a predominating class A character (Nieboer & Richardson 1980).

As shown by SEM, TEM and X-ray microanalysis, Mn was immobilized in the soredia supplied with surplus Mn in two ways: firstly, it was immobilized extracellularly in encrustations of considerable size, and, secondly, it was accumulated intracellularly in polyphosphate granules. The abilility of lichens to immobilize excess cations extracellularly has been reported in numerous studies. Oxalates of Ca, Mg, Fe, Mn, Zn, Ni, Cu, and Pb (WILSON et al. 1980; WILSON & JONES 1984; CHISHOLM et al. 1987; SARRET et al. 1998) and compounds formed out of lichen acids (i.e. norstictic and psoromic acid) and Cu (PURVIS et al. 1987, 1990) or out of melanins and U (MCLEAN et al. 1998) have been found. Pb is known to be precipitated as insoluble oxides (PbO and PbO_2) and as hydrocerrusite, i.e. a basic Pb carbonate (JONES et al. 1982). The formation of extracellular encrustations containing phosphate as the main anionic component, as found in the present study, has not been reported before. On the one hand, a reduced intracellular Mn uptake as a positive effect of this extracellular immobilization is

evident (LANGE & ZIEGLER 1963, WILSON & JONES 1984). On the other hand, extensive Mn precipitates as observed in the soredia (Fig. 2-5B), are likely to impair the mass transfer of the enclosed cells.

Polyphosphate granules are common in fungi and algae (TILLBERG et al. 1980; TURNAU et al. 1993). In lichens, they have been reported from mycobiont cells of the algal layer (CHILVERS et al. 1978; ASCASO & FORTUN 1981). In a Cd-polluted pine forest, TURNAU et al. (1993) found elevated Cd concentrations in phosphate granules of mycorrhizae of the basidiomycete *Paxillus involutus*. SICKO-GOAD & STOERMER (1979) observed the immobilization of Pb with polyphosphate granules in the diatom *Diatoma tenue*. BAXTER & JENSEN (1980) and JENSEN et al. (1982) demonstrated the accumulation of several heavy metals, including Mn, in polyphosphate granules of the cyanobacterium *Plectonema boryanum*. Increased formation of high molecular weight polyphosphate concomitant with Mn uptake is also known from *Saccharomyces carlsbergensis* (OKOROKOV et al. 1983). Heavy metal immobilization in polyphosphate granules by lichenized fungi and algae has not yet been shown. Mn complexation with phosphate leads to constant concentrations of free Mn^{2+} in a very low (nanomolar) range because of the high affinity of phosphate for Mn and its marked tendency to form insoluble complexes (KIHN et al. 1988, FARCASANU et al. 1995). However, the permanently low concentration of free Mn^{2+} in the cytoplasm is thought to cause a steady intracellular uptake driven by a concentration gradient (KIHN et al. 1988). This would imply a constant consumption of phosphate. We have no information whether Mn immobilization leads to a deficiency of intracellular soluble phosphate, but such a deficiency would severely affect cell metabolism and could be a cause for the inhibition of soredia growth on Mn-rich substrates. In order to clarify this question, measurements of the ATP content of Mn-stressed soredia should be carried out (SILBERSTEIN et al. 1990).

Significantly decreased P concentrations in the fungal cell walls concomitant with unchanged intracellular concentrations of this element in the mycobiont suggest that P from the fungal cell wall served for complexation of Mn, probably in particular in the extracellular precipitates. This may contribute to the lysis of cell walls which has been frequently observed in both the mycobiont and algal cells. In mycelia of the basidiomycete *Pisolithus arrhizus*, TURNAU & DEXHEIMER (1995) established an

enhanced acid phosphatase activity in the cell walls subsequent to exposure to Cd. Acid phosphatases are capable of releasing phosphate ions by hydrolysis of various phosphate esters (ESSNER 1973). In *Pisolithus arrhizus*, autolysis took place in centers of high acid phosphatase activity (TURNAU & DEXHEIMER 1995). Enhanced acid phosphatase activity subsequent to heavy metal exposure was also observed in *Citrobacter* (MACASKIE & DEAN 1990). The phosphate released by the enzyme was proven to precipitate stoichiometrically with the metal ions. FARCASANU et al. (1995) established mutants of *Saccharomyces cerevisiae* deficient in protein phosphatase 2B to be more sensitive to $MnCl_2$ than cells with this enzyme, as the phosphatase reduced the intracellular uptake.

In the mycobiont, Mn uptake caused significant loss of Na, Mg and Ca from the cell walls, of Ca from the cell lumina, and of Na and Mg from the polyphosphate granules, whereas in *Trebouxia*, only the Mg content of the polyphosphate granules was significantly reduced. This explains the alleviating effect Ca and Mg had on Mn toxicity in our growth experiment. On the surface of the cell walls, Ca and Mg compete with Mn for binding sites (e.g. carboxyl groups). These cation exchange sites provide a buffer between the cation supply from the environment and intracellular concentrations. They are normally occupied, primarily, by Ca, Mg and H^+ (BROWN & BECKETT 1984, BROWN & BROWN 1991). Hence, an elevated supply of Ca and Mg impedes binding of Mn to the extracellular exchange sites and, thus, also intracellular uptake. Correspondingly, high concentrations of Mn alone replace Ca and Mg as well as K and Na from the extracellular binding sites so that their concentration decreases in the cell walls and also intracellularly. A decrease of Ca in the cell walls may have contributed to their disintegration, as Ca is essential for their stabilization (MARMÉ 1983). Ca was found to alleviate Mn toxicity in the green alga *Kirchneriella lunaris* (ISSA et al. 1995). Mg reduces Mn uptake in *Saccharomyces cerevisiae* (BLACKWELL et al. 1998). Furthermore, intracellular Mg counteracts heavy metal toxicity by stimulating cellular damage-repair mechanisms, as found for Co in *S. cerevisiae* (AOYAMA et al. 1986). BLACKWELL et al. (1998) highlighted that the intracellular Mn/Mg ratio was more closely linked with cell viability in *S. cerevisiae* than the absolute intracellular Mn concentration. Remarkably, even the controls with excess Ca or Mg yielded a slightly reduced rate of grown *H. physodes*_soredia. This agrees with the preference of *H. physodes* for weakly acidic substrates (WIRTH 1995). FLETCHER (1976) established a

significantly higher replacement of extracellular K from the acidophytic *Parmelia saxatilis* than from the calciphytic *Xanthoria parietina* subsequent to Ca exposure.

Electron microscopy revealed damages to *Trebouxia* and to the mycobiont even in grown soredia of the excess Mn assay. Also in grown soredia, collapsed cells accumulated considerable amounts of Mn and P. The decisive difference between grown soredia and not grown ones was the ratio of damaged cells with elevated Mn content to intact cells. It is apparent from the occurrence of Mn accumulation and Mn-induced damage, even in grown soredia, that these macroscopically intact soredia were also affected by the supply of excess Mn. This implies that these soredia may perhaps die as well when exposed to excess Mn for a longer time than the 8 days of the experiment, as would happen in Mn-rich habitats in nature.

Aside from the limited feasibility of culture experiments with lichens, a critical point in assessing the relevance of chemical site factors for epiphytic lichens is that the availability of chemical elements in the field is hard to estimate. In general, lichens take up chemical elements from the substrate, from precipitation, and from dry deposition (KERSHAW 1985). In a study of spruce forests of the Harz Mountains in northern Germany that gave rise to the experimental work presented here, HAUCK (2000) established in a multiple correlation analysis that the Mn content in thalli of *H. physodes* was significantly affected by the Mn content of the colonized bark, but not by the Mn concentration in stemflow. BOSSERMAN & HAGNER (1981) found a significant influence of the Mn content in the substrate on the Mn content in thalli of *Parmelia* and *Usnea* species. Thus, there is no doubt that the Mn content of the substrate does affect *H. physodes*. In contrast to the element content of soil, there is no standard method for determining the availability of cations from bark. While in numerous studies, including HAUCK (2000) and HAUCK et al. (2001b), the element content of bark is specified as the total content, extracts with $SrCl_2$ or $BaCl_2$ were used for cation measurements by FARMER et al. (1991) and GOWARD & ARSENAULT (2000). However, these extractants are perhaps not appropiate for assessing the exchangeability of heavy metals from organic surfaces (BRANQUINHO et al. 1997) so that comparative tests with several extracts are necessary. An exact estimation of the amount of cations available to the lichen thallus will probably never be obtained, because, for example, different species may have different abilities for cation uptake from the same substrate. Furthermore, the

role of secondary lichen compounds for ion availability from bark has not been investigated (LAWREY 1986). Several lichen acids are known to release cations (Ca, Mg, Fe, Al) from siliceous rock (ISKANDAR & SYERS 1972; ASCASO & GALVAN 1976).

Despite the unknown amount of lichen-available Mn, our experiments suggest that the negative correlation found between cover values of *H. physodes* and the Mn content of the substrate (HAUCK 2000, HAUCK et al. 2001b) was causal. In the culture experiment, significant reduction of soredia growth already occurred at 3 mM Mn, whereas up to 11 mmol Mn kg^{-1} d. wt. were measured in bark. In stemflow measurements, LEVIA & HERWITZ (2000) established Mn to be leached from *Carya glabra* especially in winter when temperature was oscillating near freezing point. They determined that increased viscosity and surface tension of the waterfilm coating the tree surface was responsible for this. In spruce stands of the Harz Mountains, HAUCK (2000) found marked peaks in the Mn content of stemflow in winter. The peaks in Mn leaching from the substrate coincide with the season of highest soredia viability in *H. physodes* (FIECHTER & HONEGGER 1988). This infers that Mn availability is highest in a season particularly relevant to the establishment of *H. physodes*. As BAILEY (1968) showed, lichen soredia are transported despite their hydrophobic properties in water trickles. Thus, waterfilms with high Mn concentrations probably participate already in the dispersal of *H. physodes* soredia, even before they start to grow. Furthermore, ascospore discharge and germination of many lichen species peak in winter (PYATT 1969, CLAYDEN 1997). This indicates that high Mn supply of the substrate may concern not only *H. physodes*, but also the generally very low lichen diversity found on Mn-rich spruce bark in the Harz Mountains (HAUCK 2000) was possibly due to Mn toxicity. There are no studies of susceptibility of lichen ascospores to Mn, but Cu, Pb and Hg proved to inhibit ascospore germination in *Graphis scripta*, *Lecidella elaeochroma* and *Porpidia macrocarpa* (PYATT 1976).

The Mn content in spruce bark is determined by the Mn content of the soil (LÖVESTAM et al. 1990, HAUCK 2000). The latter depends on soil acidity and redox status (NORVELL 1988). Even though soil in the Harz Mountains has been strongly acidified due to anthropogenic H^+ deposition (MALESSA 1993), a variation in the Mn content and Mn/Ca, Mn/Mg and Mn/Si ratios of spruce bark is evidently caused by natural soil conditions, i.e. parent material, thickness and water relations. Hence, the Mn status of

the bark could be an important natural site parameter for epiphytic lichens, but its significance increases due to acidic deposition.

Chapter 3

Element distribution in soredia of the epiphytic lichen *Hypogymnia physodes* cultivated with manganese in a micromolar concentration

Abstract

Element distribution was studied in soredia of the epiphytic lichen *Hypogymnia physodes* cultivated for up to 11 days on a mineral agar medium with 500 µM $MnCl_2$. Mn concentrations in the cell walls, the cell lumina and the polyphosphate granules of the mycobiont were elevated after 4 days. In the photobiont (*Trebouxia jamesii*) of sucessfully grown soredia, Mn concentrations in the cell lumina and the polyphosphate granules were not altered even after 11 days. This suggests that the cell interior of the photobiont as the most sensitive part of the soredium can temporarily be protected against the uptake of toxic Mn concentrations by Mn adsorption at the fungal and algal cell walls as well as by intracellular Mn immobilization in the polyphosphate granules of the mycobiont. The ability to maintain low intracellular Mn concentrations in the photobiont differs between individual soredia. In algal cells of not grown soredia, large amounts of Mn were found to be immobilized in the polyphosphate granules. Grown and especially not grown soredia cultivated with 500 µM $MnCl_2$ for 11 days had significantly reduced K and Mg concentrations in the cell lumina of the *Trebouxia* cells. This is attributed to Mn-induced membrane damage in the photobiont, whereas no evidence was found for loss of membrane integrity in the mycobiont. Furthermore, reduced Mg/Mn ratios in soredia cultivated with 500 µM $MnCl_2$ are thought to affect the viability of the soredia. The results suggest that negative correlations between the cover of *H. physodes* and the Mn content of conifer bark found in previous studies are causal. A positive correlation between Mn and Ca concentrations in the photobiont suggests that Ca is taken up concomitant to Mn in order to maintain constant Ca/Mn ratios. This agrees with decreasing cover values of *H. physodes* with decreasing Ca/Mn ratios in

conifer bark or stemflow, which have been found repeatedly in field studies in Germany and in North America.

1. Introduction

Excess Mn has been demonstrated to be toxic to the epiphytic lichen *Hypogymnia physodes*. The growth of soredia, which are the most important diaspores of *H. physodes*, is inhibited due to surplus concentrations of Mn (HAUCK et al. 2002 d). Decreasing growth rates concomitant to increasing Mn concentrations are associated with decreasing concentrations of the chlorophylls *a* and *b*. Not yet published work shows that Fe alleviates Mn-induced growth inhibition of *H. physodes* soredia. Furthermore, Fe partly compensates for the Mn-induced reduction of the chlorophyll concentrations in *H. physodes*. These findings suggest that the induction of Fe deficiency is one cause for Mn toxicity in *H. physodes*; Fe is essential in chlorophyll biosynthesis for the conversion of protoporphyrin IX into protochlorophyllid (HORST 1988). A significant decrease of the intracellular Mg/Mn ratio with increasing Mn supply could be an additional cause for the decrease of chlorophyll concentrations (HAUCK et al. 2002 c, d). Moreover, low Mg/Mn ratios could be detrimental to *H. physodes*, because Mg is essential for the stabilization of cell structures and as the corresponding cation of biomolecules such as DNA, RNA and ATP (NIEBOER & RICHARDSON 1980).

These experimental results parallel results of field studies in coniferous forests of the Harz Mountains, northern Germany, where significant negative correlations were found between the Mn concentration of *Picea abies* bark and the cover of *H. physodes* (HAUCK et al. 2001b). HAUCK (2000) found a close correlation between the Mn concentrations in *Picea abies* bark and in thalli of *H. physodes*. This agrees with results of BOSSERMAN & HAGNER (1981), DE BRUIN & HACKENITZ (1986) and SLOOF & WOLTERBEEK (1993), who also found significant correlations between the Mn content of bark and of epiphytic lichens. Analyses of the Mn distribution in bark and in wood by DE BRUIN & HACKENITZ (1986), LÖVESTAM et al. (1990) and SLOOF & WOLTERBEEK (1993) suggest that the phorophytes take up Mn from the soil and transfer it subsequent to xylem transport into the bark. In a spruce stand of the Harz Mountains with exceptionally high Mn concentrations in the soil, HAUCK et al. (2002 a) found the most

significant (negative) correlation between cover of *H. physodes* and element concentrations in bark and stemflow with the Mn/Ca ratio in stemflow. Mn is readily leached from the foliage and from the bark by precipitation, especially at low temperatures (HAUCK 2000, LEVIA & HERWITZ 2000). Not yet published studies of our group revealed decreasing cover values of numerous epiphytic lichen species with increasing Mn/Ca ratios in the bark of coniferous forests of Montana and of an *Acer saccharum* dominated mixed hardwood forest of New York State, U.S.A. These correlations agree with experimental results, which prove that Ca and Mg reduce the extracellular adsorption and the intracellular uptake of Mn in *H. physodes* (HAUCK et al. 2002 c). Moreover, Ca and Mg compensate for the Mn-induced reduction of soredia growth in *H. physodes* (HAUCK et al. 2002 d).

Scanning electron microscopy (SEM) shows that soredia cultivated with excess Mn tend to have smaller photobiont cells (*Trebouxia jamesii* [Hildreth & Ahmadjian] Gärtner); many algal cells are collapsed (HAUCK et al. 2002 d). The photobiont cells are surrounded by a network of densely branched hyphae in control samples, whereas the fungal hyphae are shortened and swollen, and have less contact to the photobionts, in soredia on Mn-rich substrate. Extracellular encrustations on the soredia were shown to consist primarily of Mn and P by means of X-ray microanalysis. Intracellularly, Mn is accumulated in polyphosphate granules both of the mycobiont and the photobiont as shown by transmission electron microscopy (TEM) in combination with X-ray microanalysis (HAUCK et al. 2002 d).

The observations of HAUCK et al. (2002 d) were made in soredia cultivated on agar media with 7 mM $MnCl_2$ for 8 days. The availability of minerals from the bark for epiphytic lichens is insufficiently studied. This is a general problem in relating the results of experimental studies with epiphytic lichens to field data. In the Harz Mountains, total Mn concentrations between 0.3 and 11 mmol kg^{-1} d. wt. were found in *Picea abies* bark (HAUCK 2000). The percentage of available ions is difficult to assess, as the potential role of secondary metabolites in element uptake from bark is unknown. Studies with oxalic acid (BOYLE et al. 1987) and with lichen substances such as depsides and depsidones (SYERS 1969; ISKANDAR & SYERS 1972; ASCASO & GALVAN 1976) suggest that these metabolites can release cations from rock. Since we aimed at coming closer to field conditions with our Mn toxicity studies, we carried out a culture experiment with

soredia of *H. physodes* with a considerably lower $MnCl_2$ concentration compared to HAUCK et al. (2002 d). We selected an $MnCl_2$ concentration of 500 µM and tested by means of X-ray microanalysis the hypothesis that, at this concentration, Mn is taken up in significant amouts by the soredia. Furthermore, we investigated effects on the concentrations of other elements in the cell lumina, in the polyphosphate granules and in the cell walls. By comparing grown and not grown soredia, the hypothesis was tested that even at this micromolar $MnCl_2$ concentration growth inhibition is due to Mn-caused changes of element concentrations in the soredia. Moreover, samples for X-ray microanalysis were taken at two different dates, i.e., on the fourth and on the eleventh day of the experiment, in order to assess the effect of the duration of the exposure to Mn on element concentrations in the soredia.

2. Materials and methods

2.1 Soredia cultivation

Thalli of the foliose lichen *Hypogymnia physodes* (L.) Nyl. were collected the day before usage and stored in a plastic bag at ca. 5 °C at dark. Soredia were cultivated in Petri dishes with mineral agar medium. The medium (Bold's basal medium; BBM) contains 2.9 mM $NaNO_3$, 1.3 mM KH_2PO_4, 430 µM K_2HPO_4, 300 µM $MgSO_4$, 220 µM $CaCl_2$, 430 µM NaCl, 185 µM H_3BO_4, 18 µM $FeSO_4$, 31 µM $ZnSO_4$, 7 µM $MnCl_2$, 5 µM MoO_3, 6 µM $CuSO_4$, 2 µM $Co(NO_3)_3$, 130 µM Na_2EDTA, and 550 µM KOH. The pH was adjusted to 3.1 using HCl and NaOH, which corresponds to the pH found in bark and stemflow of *Picea abies* in the Harz Mountains (HAUCK 2000). $MnCl_2$ in a concentration of 500 µM was added to the medium in the assay with surplus Mn. In a preliminary test with *Picea abies* bark from the Harz Mountains, 13 ± 2 % of the total Mn were exchangeable from (not homogenized) pieces shaken in 25 mM $SrCl_2$. Since HAUCK (2000) measured total Mn concentrations of up to 11 mmol kg^{-1} d. wt. in the bark, it is plausible to assume that 500 µM is in the range of available Mn concentrations that may occur in the surroundings of mycobiont hyphae, which penetrate the bark. The availability of Mn from BBM is not known, but probably higher than from bark. Soredia cultivated on BBM at pH 3.1 without additives (i.e., with an $MnCl_2$ concentration of 7 µM) served as controls. About 500 soredia were spread over each Petri dish with forceps. The plates were stored in a growth chamber at 80 % relative humidity, a day temperature (for 13 h daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1} and a night temperature of 10 °C. Soredia were sampled for X-ray microanalysis (1) on the fourth day of incubation in the growth chamber from the 500 µM $MnCl_2$ assay, (2) on the eleventh day from the 500 µM $MnCl_2$ assay, (3) on the eleventh day from the control. On the eleventh day of cultivation, soredia on the 500 µM $MnCl_2$ plates were easy to separate in grown (green, large) ones and in not grown (whitish, small) ones (HAUCK et al. 2002 d), whereas no significant differences were observed after 4 days. Therefore, grown and not grown soredia were separately sampled from the 500 µM $MnCl_2$ plate 11 days after the start of the experiment.

2.2 Transmission electron microscopy and X-ray microanalysis

Soredia were sampled from the Petri dishes by cutting the agar into blocks of 2 x 2 mm under the dissecting microscope; in this way, three soredia of each variant — i.e., (1), (2) grown, (2) not grown, (3) — were collected and separately treated as replicate samples. The agar blocks were rapidly frozen in a 2 : 1 mixture of propane and isopentane cooled with liquid nitrogen to –196 °C, freeze-dried at –45 °C for 3 days and stored at 20 °C in a desiccator over silica gel. Freeze-dried agar blocks with soredia were infiltrated with ether in a vacuum-pressure chamber and embedded in styrene-methacrylate (FRITZ 1989). Blocks were cut with dry glass knives with an ultra-microtome in ca. 1.0 µm thick sections. The sections were mounted on adhesive-coated 100-mesh hexagonal grids (FRITZ 1991), coated with carbon and stored over silica gel. The sections were analyzed in a Philips EM 420 with the energy dispersive system EDAX DX-4 (EDAX Inc., Mahwah, New Jersey, U.S.A.). The accelerating voltage was 120 kV, the take-off angle 25° and the counting time 60 live seconds. Quantitative data (mmol dm^{-3} element content) were obtained as described by FRITZ & JENTSCHKE (1994), taking into account the calibration coefficients (Cliff-Lorimer factors) of the elements relative to K. Concentrations given in the following are based on sum spectra that were calculated from 6 - 10 spectra. The calculation of sum spectra has the advantage of lower detection limits compared to single spectra.

2.3 Statistics

Arithmetic means ± standard error are given throughout the paper. Data were tested for normal distribution with the Shapiro-Wilk test. Significance of differences between samples was tested with Duncan's multiple range test. Statistical analyses were computed with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.).

3. Results

The Mn concentrations in the cell walls, the cell lumina and in the polyphosphate granules of the mycobiont increased with increasing persistence of the exposure to 500 µM $MnCl_2$; however, the difference between soredia cultivated for 4 or 11 days was mostly not significant (Table 3-1). In the controls, Mn was not detectable in the cell lumina of the mycobiont. In the fungal cell walls, K concentrations decreased and Fe concentrations increased from the control over the 4 day variant and the grown soredia sampled after 11 days to the not grown soredia sampled after 11 days. Zn concentrations in the fungal cell walls of soredia cultivated with 500 µM $MnCl_2$ for 11 days tended to be higher compared to the control and to the soredia harvested after 4 days (Table 3-1). The ratios of Ca, Mg and Si to Mn were significantly lower in the $MnCl_2$-treated soredia than in the control (Table 3-2). The Si/Mn ratio as well as the Si concentration exhibited an insignificant trend for lower values in the fungal cell walls of not grown versus grown soredia.

In the cell lumina of the mycobiont, the concentrations of K, Ca and Mg tended to be higher after 11 days than after 4 days (Table 3-1). The K and Mg concentrations as well as the Mg/Mn and the Si/Mn ratios had a trend for higher values in the fungal cell lumina of grown versus not grown soredia. After 11 days, Ca concentrations had a tendency for higher values in the fungal cell lumina of Mn-exposed soredia compared to the control. Aside from the Mn content, little variation occurred between the different treatments in the element concentrations of the fungal polyphosphate granules. Ca had an insignificant trend for increasing concentrations from the 4 days variant over the soredia treated for 11 days with 500 µM $MnCl_2$ to the control. Na concentrations were higher in the $MnCl_2$-treated soredia at the fourth day than at the eleventh day. The ratios of Ca, Mg and Si to Mn were higher in the controls than in the soredia supplied with 500 µM $MnCl_2$; however, this was only significant in the case of the Mg/Mn ratio (Table 3-2).

Table 3-1. X-ray microanalysis (TEM) of mycobiont cells in *Hypogymnia physodes* soredia cultivated with 500 µM $MnCl_2$[a]

	Control		4 days		11 days; grown		11 days; not grown	
Cell walls (*n* = 4; 3; 3; 3):								
Mn	0.55 ± 0.11	a	6.58 ± 0.87	b	9.68 ± 3.48	b	11.2 ± 1.9	b
K	205 ± 10	a	180 ± 15	ab	164 ± 18	ab	151 ± 19	b
Ca	15.7 ± 1.8	a	11.4 ± 1.81	a	15.1 ± 4.3	a	16.5 ± 3.1	a
Mg	16.7 ± 2.2	a	4.80 ± 0.68	a	14.8 ± 4.6	a	10.4 ± 5.5	a
Fe	1.67 ± 0.11	a	1.99 ± 0.13	a	2.32 ± 0.54	ab	3.99 ± 1.13	b
Si	24.6 ± 7.0	a	132 ± 106	a	31.0 ± 12.0	a	20.1 ± 2.1	a
P	93.8 ± 11.6	a	57.1 ± 7.7	a	64.2 ± 20.2	a	74.2 ± 19.4	a
S	53.5 ± 7.6	ab	59.0 ± 6.8	a	36.3 ± 6.4	ab	34.3 ± 5.6	b
Cl	95.1 ± 5.6	a	155 ± 4	a	112 ± 25	a	116 ± 45	a
Cell lumina (*n* = 4; 3; 3; 3):								
Mn	0.00 ± 0.00	a	2.57 ± 0.66	b	5.61 ± 0.94	c	4.80 ± 1.09	bc
K	77.7 ± 9.5	ab	53.6 ± 19.7	b	110 ± 7	a	81.0 ± 12.4	ab
Ca	4.62 ± 1.6	a	2.21 ± 1.33	a	6.22 ± 2.32	a	6.21 ± 1.59	a
Mg	13.3 ± 0.5	a	5.65 ± 1.67	b	18.6 ± 2.2	c	8.39 ± 0.19	b
Fe	1.36 ± 0.22	a	2.11 ± 0.37	a	1.69 ± 0.25	a	2.08 ± 0.49	a
Si	13.7 ± 4.1	ab	41.2 ± 17.9	a	18.0 ± 5.8	ab	9.35 ± 2.37	b
P	90.2 ± 10.2	a	58.7 ± 20.9	a	108 ± 17	a	83.2 ± 9.3	a
S	50.4 ± 5.5	a	46.0 ± 14.3	a	55.6 ± 7.5	a	44.5 ± 2.4	a
Cl	35.3 ± 5.1	a	49.4 ± 21.1	a	69.3 ± 9.1	a	46.3 ± 5.3	a
Polyphosphate granules (*n* = 4; 3; 3; 3):								
Mn	5.38 ± 0.87	a	39.2 ± 6.8	b	50.1 ± 17.6	b	52.0 ± 8.6	b
P	1710 ± 97	a	1880 ± 114	a	1521 ± 87	a	1740 ± 297	a

[a] Concentrations in mmol dm^{-3}. Arithmetic means ± standard error. *n*: total of sum spectra used for control; 4 days harvest; 11 days harvest of grown soredia; 11 days harvest of not grown soredia. Each sum spectrum is computed of 9 - 10 (cell walls), 6 - 9 (cell lumina) and 7 - 9 single spectra (polyphosphate granules). Control: grown soredia cultivated for 11 days with 7 µM $MnCl_2$. Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 9$).

Cell walls, cell lumina and polyphosphate granules in the *Trebouxia* cells of all soredia supplied with 500 µM $MnCl_2$ contained more Mn than the control samples; this difference was significant for the cell walls (Table 3-3). Mn concentrations in the cell walls and in the polyphosphate granules were significantly higher in not grown soredia cultivated for 11 days with 500 µM $MnCl_2$ compared to the other variants. The Mg concentrations as well as the ratio of Mg to Mn were significantly higher in the algal cell

walls of grown than of not grown soredia (Tables 3-2, 3-3). Irrespective of the $MnCl_2$ supply, the Mg concentrations in the algal cell walls were higher after 11 days than after 4 days of incubation (Table 3-3). The concentrations of Si and P as well as the Si/Mn ratio had a trend for higher values in the algal cell walls of grown than of not grown $MnCl_2$-exposed soredia. S concentrations were higher in the algal cell walls of soredia cultivated for 11 days with 500 µM $MnCl_2$ compared to soredia sampled after 4 days or compared to the control. The highest Ca/Mn and Mg/Mn ratios were found in the algal cell walls of the controls.

In the cell lumina of the photobiont, significantly lower K concentrations were found in the soredia cultivated for 11 days with 500 µM $MnCl_2$ compared to those sampled after 4 days and to the control samples (Table 3-3). Moreover, there was an insignificant trend that the *Trebouxia* cell lumina of grown soredia contained more K compared to not grown soredia. Soredia sampled after 11 days contained more Mg and less Fe in the algal cell lumina than soredia cultivated for 4 days. In contrast to the other samples, Zn was not detectable in the algal cell lumina of $MnCl_2$-treated soredia sampled after 11 days; this applied to the algal polyphosphate granules as well. P occurred in lower concentrations in the cell lumina of $MnCl_2$-exposed photobionts on the eleventh than on the fourth day. Furthermore, there was a trend to lower concentrations in not grown versus grown soredia. The S concentrations were significantly lower and the ratios of Mg and Si to Mn insignificantly lower in algal cell lumina of not grown soredia compared to the other treatments (Tables 3-2, 3-3). In the polyphosphate granules of the photobiont, K, Mg, P and Cl concentrations in not grown soredia were reduced compared to the control; Ca concentrations in not grown soredia were much higher than in grown soredia cultivated with 500 µM $MnCl_2$ for 11 days. Concentrations Si and S as well as the Mg/Mn and the Si/Mn ratios tended to be higher in the polyphosphate granules of grown compared to not grown soredia; the Mg/Mn ratio of the control was higher than of all $MnCl_2$ treatments.

Table 3-2. X-ray microanalysis (TEM) of photobiont cells (*Trebouxia jamesii*) in *Hypogymnia physodes* soredia cultivated with 500 µM $MnCl_2$[a]

	Control		4 days		11 days; grown		11 days; not grown	
Cell walls (*n* = 4; 4; 3; 4):								
Mn	0.41 ± 0.24	a	2.71 ± 0.27	b	3.02 ± 0.80	b	5.43 ± 0.76	c
K	187 ± 18	a	184 ± 31	a	147 ± 34	a	131 ± 22	a
Ca	5.32 ± 1.72	ab	4.01 ± 1.06	ab	2.83 ± 1.21	a	8.33 ± 1.64	b
Mg	11.8 ± 3.5	a	4.00 ± 2.51	ab	10.2 ± 1.82	ab	1.95 ± 0.99	b
Fe	1.23 ± 0.20	a	1.50 ± 0.55	a	1.08 ± 0.14	a	1.09 ± 0.12	a
Si	20.5 ± 3.5	a	45.0 ± 21.4	a	52.9 ± 22.5	a	28.3 ± 6.88	a
P	92.6 ± 20.4	a	62.5 ± 23.3	a	67.6 ± 18.0	a	35.8 ± 13.2	a
S	50.9 ± 3.8	a	51.1 ± 8.9	a	26.1 ± 9.7	b	22.7 ± 6.8	b
Cl	95.4 ± 12.7	a	191 ± 8	b	76.0 ± 23.6	a	124 ± 17	a
Cell lumina (*n* = 4; 3; 3; 4):								
Mn	0.08 ± 0.08	a	0.88 ± 0.17	a	0.54 ± 0.05	a	1.04 ± 0.52	a
K	113 ± 12	a	117 ± 22	a	67.7 ± 14.6	b	39.0 ± 9.1	b
Ca	0.54 ± 0.27	a	1.35 ± 0.78	a	0.22 ± 0.22	a	1.10 ± 0.39	a
Mg	17.3 ± 2.5	ab	37.6 ± 14.9	a	12.5 ± 1.32	b	4.13 ± 2.53	b
Fe	2.27 ± 0.10	a	3.38 ± 0.29	b	2.20 ± 0.13	a	1.80 ± 0.29	a
Si	8.89 ± 1.27	a	20.5 ± 9.2	a	14.9 ± 4.6	a	8.28 ± 1.26	a
P	53.7 ± 5.8	ab	98.0 ± 35.7	a	31.7 ± 2.3	b	22.4 ± 4.36	b
S	76.2 ± 6.1	a	118 ± 22	b	41.4 ± 10.9	ab	26.2 ± 3.86	c
Cl	36.5 ± 4.3	a	74.4 ± 43.7	a	24.7 ± 11.5	a	17.0 ± 5.4	a
Polyphosphate granules (*n* = 4; 4; 3; 5):								
Mn	0.28 ± 0.16	a	5.35 ± 2.90	a	2.86 ± 0.36	a	69.8 ± 21.8	b
P	1110 ± 233	a	842 ± 241	ab	745 ± 95	ab	462 ± 47	b

[a] Confer Table 3-1. Each sum spectrum is computed of 8 - 10 (cell walls), 6 - 9 (cell lumina) and 7 - 9 single spectra (polyphosphate granules). Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, df = 11 [cell walls], 10 [cell lumina], 12 [polyphosphate granules]).

4. Discussion

Mn concentrations that increase in the soredia of *H. physodes* within 4 days show that Mn is readily taken up from agar plates with 500 µM $MnCl_2$. The fast adsorption agrees with studies of the Mn uptake in thalli of *H. physodes* from solution (HAUCK et al. 2002 c) as well as with the studies of, e.g., SLOOF & WOLTERBEEK (1993) and HAUCK (2000) that suggest Mn uptake from the substrate in lichens. The significantly elevated Mn concentrations after 4 days in all fungal compartments (i.e., cell walls, cell lumina, and polyphosphate granules) and in the algal cell walls, but not in the cell lumina and in the

polyphosphate granules of the *Trebouxia* photobiont suggest that the intracellular space of the algal cells as the most sensitive part of the lichen symbiosis is not directly exposed to temporarily and moderately elevated Mn concentrations. Rather, most Mn is allocated in the fungal cell walls and in intracellular polyphosphate granules of the mycobiont cells. As neither Mn-containing crystals nor amorphous encrustations of Mn phosphates were observed, most extracellular Mn can be supposed to be bound to cation exchange sites in the fungal cell walls (BROWN & BROWN 1991; RICHARDSON 1995). In soredia of *H. physodes* cultivated with 7 mM $MnCl_2$ for 8 days, HAUCK et al. (2002 d) found substantial extracellular encrustations primarily consisting of Mn and P. The intracellular immobilization in polyphosphate granules was also observed in that study.

After 11 days of exposure to 500 µM $MnCl_2$, the algal cells of some soredia are still protected against the intrusion of significant amounts of Mn; these soredia are able to grow. Other soredia apparently fail to protect the cell interior of their photobiont cells against Mn and lose the ability to grow. The Mn concentration in the polyphosphate granules of soredia that were not able to grow was about 25 times as much as in sucessfully established soredia. The causes for the individual differences in the resistance of the soredia of *H. physodes* to a given application rate of Mn may depend on individual differences in the element content and in the physiological status of the soredia. In the field, microclimatic differences may also contribute to differences in the Mn resistence of the soredia. The rate of grown to not grown *H. physodes* soredia rapidly decreases with increasing Mn content of the substrate (HAUCK et al. 2002 d).

Table 3-3. Molar ratios of Ca, Mg, Fe and Si to Mn in mycobiont and photobiont cells of *Hypogymnia physodes* soredia grown with 500 µM $MnCl_2$[a]

	Control		4 days		11 days; grown		11 days; not grown	
Mycobiont, cell walls (*df* = 9):								
Ca/Mn	34.9 ± 12.3	a	1.72 ± 0.05	b	1.65 ± 0.28	b	1.47 ± 0.06	b
Mg/Mn	33.0 ± 5.3	a	0.74 ± 0.09	b	1.62 ± 0.33	b	1.18 ± 0.80	b
Fe/Mn	0.01 ± 0.00	a	0.00 ± 0.00	a	0.01 ± 0.00	ab	0.03 ± 0.01	b
Si/Mn	44.9 ± 9.3	a	25.2 ± 21.7	ab	4.65 ± 2.10	b	1.86 ± 0.23	b
Mycobiont, cell lumina (*df* = 6):								
Ca/Mn	-[b]		0.92 ± 0.46	a	1.05 ± 0.21	a	1.28 ± 0.18	a
Mg/Mn	-[b]		2.22 ± 0.45	a	3.51 ± 0.69	a	1.93 ± 0.42	a
Fe/Mn	-[b]		0.06 ± 0.03	a	0.02 ± 0.00	a	0.03 ± 0.00	a
Si/Mn	-[b]		23.0 ± 13.6	a	3.24 ± 1.06	a	2.36 ± 1.05	a
Photobiont, cell walls (*df* = 9):								
Ca/Mn	9.09 ± 2.74	a	1.46 ± 0.34	b	0.92 ± 0.24	b	1.55 ± 0.23	b
Mg/Mn	10.3 ± 0.21	a	1.36 ± 0.76	bc	4.35 ± 1.91	c	0.57 ± 0.21	b
Fe/Mn	0.01 ± 0.00	a	0.01 ± 0.00	a	0.01 ± 0.00	a	0.02 ± 0.00	a
Si/Mn	24.0 ± 6.1	a	16.8 ± 8.5	a	22.5 ± 11.2	a	5.40 ± 1.44	a
Photobiont, cell lumina (*df* = 6):								
Ca/Mn	0.00[c]	a	1.38 ± 1.52	a	0.42 ± 0.42	a	0.84 ± 0.22	a
Mg/Mn	56.4[c]	a	53.3 ± 26.8	a	23.2 ± 2.70	a	8.38 ± 4.19	a
Fe/Mn	0.02[c]	a	0.03 ± 0.00	a	0.04 ± 0.01	a	0.06 ± 0.02	a
Si/Mn	26.2[c]	a	24.6 ± 10.6	a	27.9 ± 9.8	a	7.53 ± 2.26	a

[a] Arithmetic means ± standard error. Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$). [b] Mn concentration 0.00 ± 0.00 mmol dm^{-3}. [c] Only one replicate with an Mn concentration > 0.00 mmol dm^{-3}.

A decisive cause for the susceptibility of the soredia to Mn, which is deducible from the data of the X-ray microanalysis, may be membrane damage of the algal cells. The cell lumina of not grown soredia contained only about one third of the corresponding K concentration in the control; the K concentrations in the cell lumina of grown soredia sampled after 11 days were about half as high as in the control. Since the K concentrations in the polyphosphate granules of the *Trebouxia* cells of not grown soredia were also lower than in the control, the lower intracellular K concentrations can be attributed to the loss of membrane integrity. Likewise reduced intracellular Mg concentrations in the photobiont support this explanation; loss of Mg from the cell interior concomitant to loss of K is seen as an indicator of severe membrane damage (BROWN & BROWN 1991; BRANQUINHO et al. 1997). The occurrence of membrane damage in the photobiont of *H.*

physodes soredia due to Mn is apparently dependend on the duration of the Mn exposure, because the cell lumina of algal cells in soredia cultivated for 4 days had not reduced K and Mg concentrations. The Mn concentrations may be less relevant, as the concentrations of K and Mg in the algal cell lumina of *H. physodes* soredia were not reduced subsequent to cultivation for 8 days with 7 mM $MnCl_2$ (HAUCK et al. 2002 d).

The intracellular K and Mg concentrations of the mycobiont in soredia cultivated for 11 days with 500 µM $MnCl_2$ were not significantly different from those in the control. This suggests that the fungal cells did not suffer from membrane damage. This result agrees with results of HAUCK et al. (2002 c) that no efflux of intracellular K or Mg was detectable when thallus pieces of *H. physodes* were exposed to an $MnCl_2$ concentration as high as 100 mM for up to 2 h. HAUCK et al. (2002 c) applied like many others in similar studies the sequential extraction procedure of BROWN & BECKETT (1984). In this procedure, free apoplastic ions are eluted with water, and extracellularly bound cations are subsequently exchanged with $NiCl_2$ or (modified for class B cations with high affinity for the exchange sites) with EDTA (BRANQUINHO & BROWN 1994). The intracellular ions are determined in an acid digest of the residual sample. In other studies, chemical analysis was limited to the K concentration in the incubation medium (BURTON et al. 1981). BECKETT & BROWN (1984) already emphasized that both procedures more easily detect reactions of the mycobiont, as it constitutes the major part of the biomass in a lichen thallus. The present data proves for the first time the occurrence of membrane damage restricted to the photobiont. This infers that data obtained by chemical analyses of the incubation medium or of solutions obtained by sequential extraction should generally only be used for discussing the membrane status of the mycobiont, as these methods are not suited for detecting efflux of K or Mg that is limited to the algal cells. Significantly higher K and Mg concentrations in the fungal cell lumina of grown soredia collected after 11 days compared to soredia cultivated for 4 days reflect an apparently effective uptake of these ions by the mycobiont from the substrate. This agrees with results of a not yet published experiment of our group, which suggest that *H. physodes* adsorbs K rather from the substrate than from precipitation.

Reduced Mg/Mn ratios are probably a further cause for the lacking ability of a proportion of the soredia to grow. HAUCK et al. (2002 c) suggested that lower intracellular Mg/Mn ratios in *H. physodes* than in the crustose lichen *Lecanora conizaeoides*

could cause the higher Mn tolerance of the latter. Decreasing intracellular Mg/Mn ratios also occurred in thalli of the epiphytic lichens *Alectoria sarmentosa* and *Bryoria fuscescens* subsequent to short-term incubation in $MnCl_2$ solution (HAUCK & PAUL unpubl.). In *Saccharomyces cerevisiae* as well as in vascular plants, the Mg/Mn ratio was found to be more significant for viability than the absolute Mn concentration (GOSS & CARVALHO 1992; BLACKWELL et al. 1997). The present element data give no evidence of an Mn-induced Fe deficiency in the soredia. This agrees with HAUCK et al. (2002 d), who even found higher Fe concentrations in the algal cell lumina of *H. physodes* soredia cultivated with 7 mM $MnCl_2$ compared to control samples. Despite this, unpublished results of our group proved an alleviation of Mn-induced growth inhibition in soredia of *H. physodes* due to $FeCl_3$. Therefore, intracellular Fe deficiency cannot be ruled out as a cause for the reduced soredia viability at 500 µM $MnCl_2$, as total and available Fe concentrations are generally poorly correlated in plant tissues (BOUMA 1983). Chlorophyll concentrations, which were established to be reduced by excess Mn in soredia of *H. physodes* by HAUCK et al. (2002 d), have not been investigated in the present study.

The trend for higher Si/Mn ratio in grown than in not grown soredia could also be significant for the Mn tolerance of the *H. physodes* soredia. HAUCK et al. (2002 d) found significantly lower Si/Mn ratios in the cell walls of collapsed versus intact *Trebouxia* cells in *H. physodes* soredia cultivated with 7 mM $MnCl_2$. Si is known to alleviate Mn toxicity symptoms in vascular plants (EL-JAOUAL & COX 1998). However, the mechanisms assumed for vascular plants (i.e., reduced Mn uptake, altered microdistribution of Mn in the plant tissue and chelation of Mn) may not necessarily apply to lichens, for which the role of Si has not been studied so far. The lower P concentrations in the cell lumina of the *Trebouxia* cells cultivated with 500 µM $MnCl_2$ for 11 versus 4 days is probably due to the consumption of P by the formation of insoluble complexes with Mn in the polyphosphate granules. HAUCK et al. (2002 d) hypothesized that Mn immobilization could lead to intracellular P deficiency in *H. physodes*. However, not yet published results of our group showed that ATP concentrations in thallus pieces of *H. physodes* were not reduced due to incubation with up to 10 mM $MnCl_2$ for 1 h; this indicates the absence of intracellular P deficiency in that case. The apparent lack of P deficiency in spite of the consumption of large amounts of P for Mn immobilization may be due to the high uptake efficacy of *H. physodes* for P from low-concentrated solutions (FARRAR 1976).

The concentrations of Ca were significantly higher in the algal polyphosphate granules of not grown soredia compared to grown soredia. In general, the Ca concentration increased with increasing Mn concentration in the photobiont (cell walls: $r = 0.52$, $P \leq 0.05$; cell lumina: $r = 0.55$, $P \leq 0.05$; polyphosphate granules: $r = 0.94$, $P \leq 0.001$). Similar observations made TURNAU et al. (1994), who found increased Ca levels in mycelia of the basidiomycete *Pisolithus arrhizus* exposed to Cd dust. In the aquatic moss *Fontinalis antipyretica*, increasing Ca concentrations were observed along with increasing Mn, Ni and Cu concentrations, when samples from differently heavy metal polluted stream waters were compared (SÉRGIO et al. 2000). Since field observations suggest that the ratio of Mn to Ca is significant for the abundance of epiphytic lichen species including *H. physodes* (HAUCK et al. 2002 a), increased Ca uptake concomitant to increasing Mn concentrations could be a stress response to stabilize the intracellular Ca/Mn ratio. Indeed, the Ca/Mn ratios were not significantly different between grown and not grown soredia cultivated with 500 µM for 11 days in spite of significantly increased Mn concentrations in algal cell walls and polyphosphate granules of not grown soredia.

5. Conclusions

While HAUCK et al. (2002 d) found significantly reduced rates of soredia growth at 3 mM $MnCl_2$ and higher, the present study shows the uptake of significant amounts of Mn in *H. physodes* soredia already at a concentration of 500 µM. Furthermore, after 11 days, but not after 4 days, the photobiont cells of soredia cultivated with 500 µM $MnCl_2$ lost the integrity of their cytoplasma membranes as indicated by significant loss of intracellular K and Mg. These observations show that, even at this relatively low $MnCl_2$ concentration, soredia are significantly affected by Mn. Longer exposure to surplus Mn than for 11 days might result in lethal damage even of soredia that were able to grow with 500 µM $MnCl_2$ in the present experiment. The results support the hypothesis that negative correlations found in the field between the Mn supply and the cover of epiphytic lichen species including *H. physodes* are due to the sensitivity of these lichen species to excess Mn (HAUCK et al. 2001 b). The positive correlation between the concentrations of Ca and Mn in the photobiont suggests that decreasing cover values of *H.*

physodes and other epiphytic lichens with decreasing Ca/Mn ratios in stemflow or bark (HAUCK et al. 2002 a) are causal. It is plausible that low Ca/Mn ratios in the environment limit the ability of *H. physodes* to maintain constant Ca/Mn ratios in the lichen thallus by Ca uptake.

Chapter 4

Ultrastructural changes in soredia of the epiphytic lichen *Hypogymnia physodes* cultivated with manganese

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Abstract

The ultrastructure of soredia of the foliose epiphytic lichen *Hypogymnia physodes* cultivated on agar plates for 11 days with three different concentrations of $MnCl_2$ (7 µM [control], 500 µM, 7 mM) was compared by means of transmission electron microscopy. Photobiont cells (*Trebouxia jamesii*) of Mn-exposed soredia had swollen and contracted thylakoids. The chloroplasts were reduced in size, and numerous lysosome-like vesicles occurred in cells with degenerated chloroplast. At 500 µM, autospores of *T. jamesii* were more severely damaged than vegetative cells. At 7 mM, autospore formation was strongly reduced. Concentric bodies and mesosome-like structures were nearly completely absent from mycobiont cells of Mn-treated soredia at both 500 µM and 7 mM. At 500 µM, the photobiont of not grown soredia was more severely damaged compared to grown soredia (defined as soredia where division of algal cells had taken place). At 7 mM, ultrastructural damage was observed in both grown and not grown soredia. This suggests that even soredia that survived the first days of cultivation are probably not capable of forming thalli on Mn-rich substrates. The ultrastructural changes in Mn-exposed soredia support the hypothesis that high Mn concentrations in bark or stemflow are a limiting factor for the abundance of *H. physodes* and other epiphytic lichens in coniferous forests of Europe and North America.

1. Introduction

The abundance of epiphytic lichens has been found to decrease with increasing Mn concentrations in bark or stemflow in coniferous forests of Europe and North America (HAUCK et al. 2001 b, 2002a, HAUCK & SPRIBILLE 2002, SCHMULL et al. 2002, SCHMULL & HAUCK 2003a). Mn is deposited in the bark after root uptake, xylem transport and radial translocation (LÖVESTAM et al. 1990; SLOOF & WOLTERBEEK 1993) and, thus, varies with the availability of Mn from the soil (HAUCK et al. 2002a). Hence, Mn is a natural site parameter, which may have become more effective at sites with soils acidified by atmospheric pollution. Mn in stemflow primarily derives from leaching from the foliage and from the bark (LEVIA & HERWITZ 2000).

Experiments carried out with the foliose lichen *Hypogymnia physodes* also suggest that excess concentrations of Mn limit the abundance of epiphytic lichens. Mn reduced the chlorophyll content of thalli and of soredia in this species (HAUCK et al. 2002d, 2003). Scanning electron microscopy (SEM) showed that fungal hyphae in *H. physodes* soredia cultivated on Mn-rich agar medium were swollen and shortened (HAUCK et al. 2002d). Moreover, the physical contact to the photobiont was strongly reduced. Photobiont cells of Mn-treated soredia were smaller and partly collapsed. Transmission electron microscopy (TEM) showed that Mn was immobilized intracellularly in polyphosphate granules both of the mycobiont and the photobiont and extracellularly in phosphate encrustations (HAUCK et al. 2002d). These phosphate encrustations completely surrounded some algal cells leading to their death. Ca, Mg and Fe were found to reduce Mn uptake and to alleviate Mn toxicity in *H. physodes* (HAUCK et al. 2002c, d, 2003).

In the present paper, ultrathin sections of *H. physodes* soredia were investigated by means of TEM in order to test the hypothesis that Mn causes damage in this species on the subcellular level. These studies were carried out in order to obtain more information about the mechanisms causing Mn toxicity in epiphytic lichens. Acidic solutions containing $CuSO_4$ and $NiSO_4$ as well as SO_2 have already been shown to cause ultrastructural damage in epiphytic lichens (HOLOPAINEN & KÄRENLAMPI 1984, TARHANEN 1998).

2. Materials and methods

2.1. Soredia cultivation

Thalli of the foliose lichen *Hypogymnia physodes* (L.) Nyl. (photobiont: *Trebouxia jamesii* (Hildreth and Ahmadjian) Gärtner) were collected the day before usage and stored in a plastic bag at ca. 5 °C at dark. Soredia were cultivated in Petri dishes with mineral agar medium. The medium (Bold's basal medium; BBM) contains 2.9 mM $NaNO_3$, 1.3 mM KH_2PO_4, 430 µM K_2HPO_4, 300 µM $MgSO_4$, 220 µM $CaCl_2$, 430 µM NaCl, 185 µM H_3BO_4, 18 µM $FeSO_4$, 31 µM $ZnSO_4$, 7 µM $MnCl_2$, 5 µM MoO_3, 6 µM $CuSO_4$, 2 µM $Co(NO_3)_3$, 130 µM Na_2EDTA, 550 µM KOH, and 18 g/l agar. Prior to the addition of agar the pH was adjusted to 3.1 using HCl and NaOH, which corresponds to the pH found in bark and stemflow of *Picea abies* in the Harz Mountains, northern Germany, where Mn was found to be a relevant site factor for *H. physodes* (HAUCK 2000). $MnCl_2$ in a concentration of 500 µM or 7 mM was added to the medium in the assays with surplus Mn. The concentration of 7 mM was already applied by HAUCK et al. (2002c) investigating structural changes and element concentrations in *H. physodes* soredia with SEM and, in semithin sections, with TEM and X-ray microanalysis. This concentration corresponded to the range of total Mn concentrations (up to 11 mmol kg^{-1} dry weight) found in *Picea abies* bark in the Harz Mountains. The availability of Mn from spruce bark or from agar medium is difficult to assess. Comparing different extraction procedures, SCHMULL & HAUCK (2003b) found that Mn is readily available compared to most other cations. In a preliminary test with *Picea abies* bark from the Harz Mountains, 13 ± 2 % of the total Mn were exchangeable from (not homogenized) pieces shaken in 25 mM $SrCl_2$. The concentration of 500 µM was chosen, because these extraction experiments suggest that, at least, this concentration is in the range of available Mn concentrations that may occur in the surroundings of mycobiont hyphae, which penetrate the bark of *Picea abies* in the Harz Mountains. The availability of Mn from BBM is not known, but probably higher than from bark. Soredia cultivated on BBM at pH 3.1 without additives (i.e., with an $MnCl_2$ concentration of 7 µM) served as controls. About 500 soredia were spread over each Petri dish with forceps. The plates were stored in a growth chamber at 80 % relative humidity, a day temperature (for 13 h daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1} and a night temperature of 10 °C for 11 days.

2.2. Sample preparation and transmission electron microscopy

Soredia were sampled from the Petri dishes (one plate per Mn concentration) by cutting the agar into blocks of 2 x 2 mm under the dissecting microscope. These blocks contained single soredia. From each Mn concentration grown and not grown soredia were separately sampled (MARGOT 1973, HAUCK et al. 2002d). Grown soredia were defined as soredia where division of algal cells took place; they differ in size and in colour (green in grown soredia versus whitish) from not grown soredia. Three agar blocks with grown or not grown soredia, respectively, were separately collected from each Mn concentration under the dissecting microscope. Samples were prefixed in 3 % glutaraldehyde in 50 mM phosphate buffer at pH 7.0 and 4 °C (HOLOPAINEN & KÄRENLAMPI 1984). After prefixation samples were washed four times with phosphate buffer, postfixed with 2 % OsO_4 and washed once with phosphate buffer. Samples were dehydrated in a graded ethanol series and embedded in epoxy resin (SPURR 1969). Ultrathin sections were cut with glass knives with an Ultracut 2 microtome (Reichert-Jung, Wien, Austria). Cuts used for electron microscopy were 90 - 150 nm thick; thickness was estimated with help of their interference colors, i.e, golden cuts were selected (FLEGLER et al. 1995). Samples were mounted on copper grids and stained with 7 % uranyl acetate in H_2O (60 min) and 2 % lead citrate (8 min; REYNOLDS 1963). The sections were studied with a Philips EM 420 using an accelerating voltage of 120 kV.

3. Results

In the control samples (Fig. 4-1), the photobiont cells were nearly completely filled with the chloroplast. In the center, the photobiont contained a single pyrenoid with numerous electron-dense pyrenoglobuli; lysosome-like vesicles were not common. Aside from vegetative cells of *Trebouxia jamesii*, the formation of autospores was frequently observed. In the mycobiont, mesosome-like structures and concentric bodies were regularly observed in the control samples. There was no significant difference in the ultrastructure of fungal and algal cells from grown versus not grown soredia of the control. The size of the chloroplast was increasingly reduced in soredia cultivated with Mn in the order 500 µM, grown < 500 µM, not grown < 7 mM grown, 7 mM grown (Figs. 4-2, 4-3). The thylakoid membranes of Mn-treated samples were swollen and contracted. This damage was stronger in not grown soredia at 500 µM Mn and in soredia cultivated with 7 mM Mn compared to grown soredia at 500 µM Mn, whereas no differences were observed between grown and not grown soredia at 7 mM Mn. At places where the chloroplast was disintegrated, lysosome-like vesicles occurred in the cytoplasm containing dark ring-like structures. At 7 mM Mn, the formation of autospores was more or less completely inhibited. At 500 µM, autospores were frequent, but suffered more damage compared to vegetative algal cells. Chloroplasts of autospores were more reduced and contained more lysosome-like vesicles. In some mitochondria, the swelling of cristae was observed in photobiont cells at 7 mM Mn. Tufts of cytoplasm were frequent on the surface of algal protoplasts bordering on mycobiont hyphae; these structures were apparently not affected by Mn. The mycobiont was equally affected by either Mn treatment. Mesosome-like structures and concentric bodies were nearly completely absent from fungal hyphae of Mn-exposed soredia. In Mn-exposed soredia, electron-dense intracellular deposits occurred in the mycobiont.

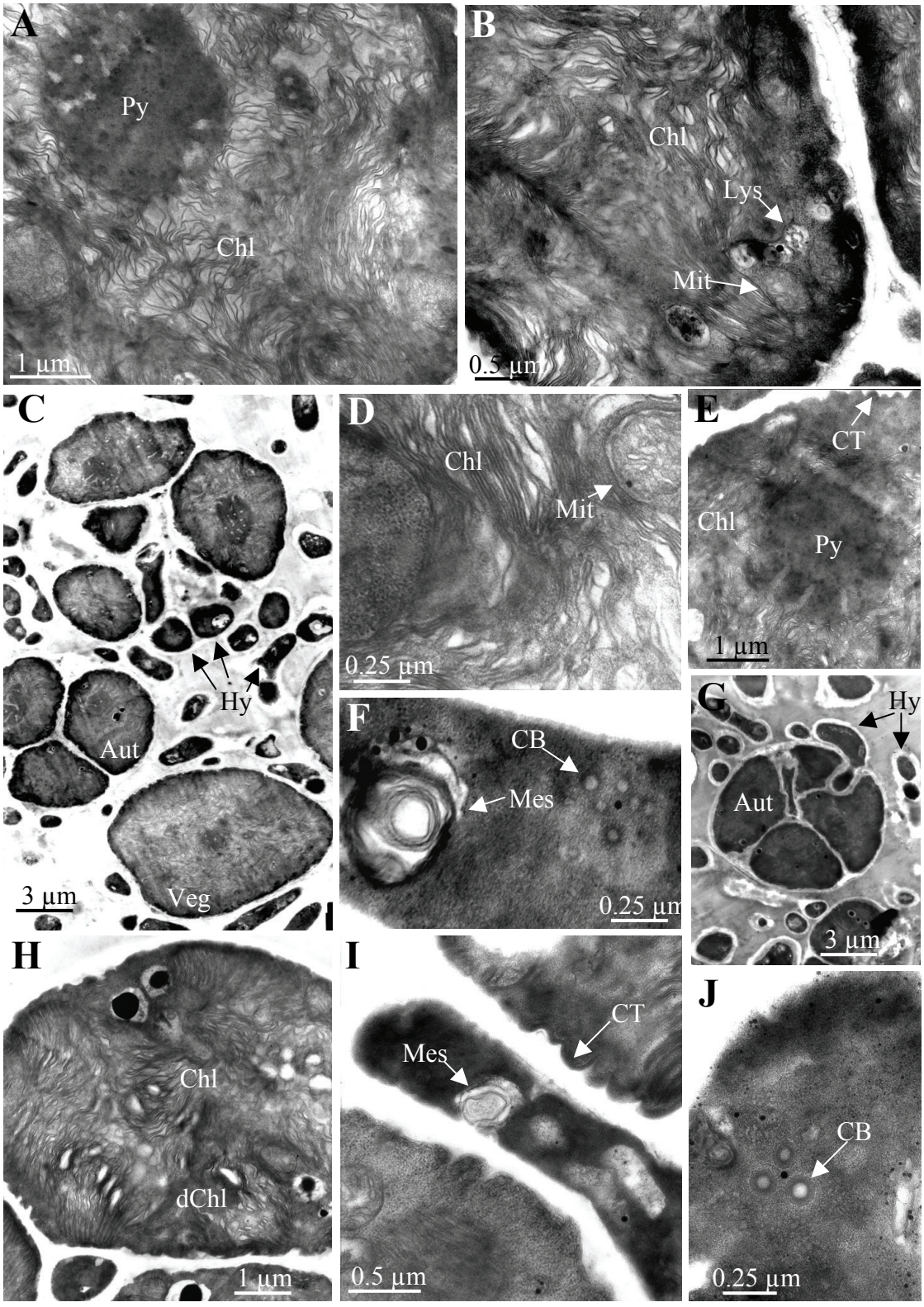

Fig. 4-1. TEM micrographs of ultrathin sections of soredia of *H. physodes* of the control, showing grown (A - D, F) or not grown soredia (E, G - J). A. Vegetative cell of *Trebouxia jamesii* more or less completely filled with the chloroplast (Chl) and with large central pyrenoid (Py). B. Autospore of *T. jamesii* filled with the chloroplast and sporadic occurrence of lysosome-like vesicles (Lys); Mit: mitochondrion. C. Assembly of soredium with mycobiont hyphae (Hy), vegetative cells of *T. jamesii* (Veg) and autospores of *T. jamesii* (Aut). D. Intact thylakoids and mitochondrion in vegetative photobiont cell. E. Vegetative photobiont cell with pyrenoid and intact chloroplast; CT: cytoplasma tufts. F. Mycobiont hypha with mesosome-like structure (Mes) and concentric bodies (CB). G. Autospores of *T. jamesii* surrounded by mycobiont hyphae. H. Autospore with partly damaged chloroplast (dChl). I. Hypha with mesosome-like structure and photobiont autospores with cytoplasma tufts. J. Hypha with concentric bodies.

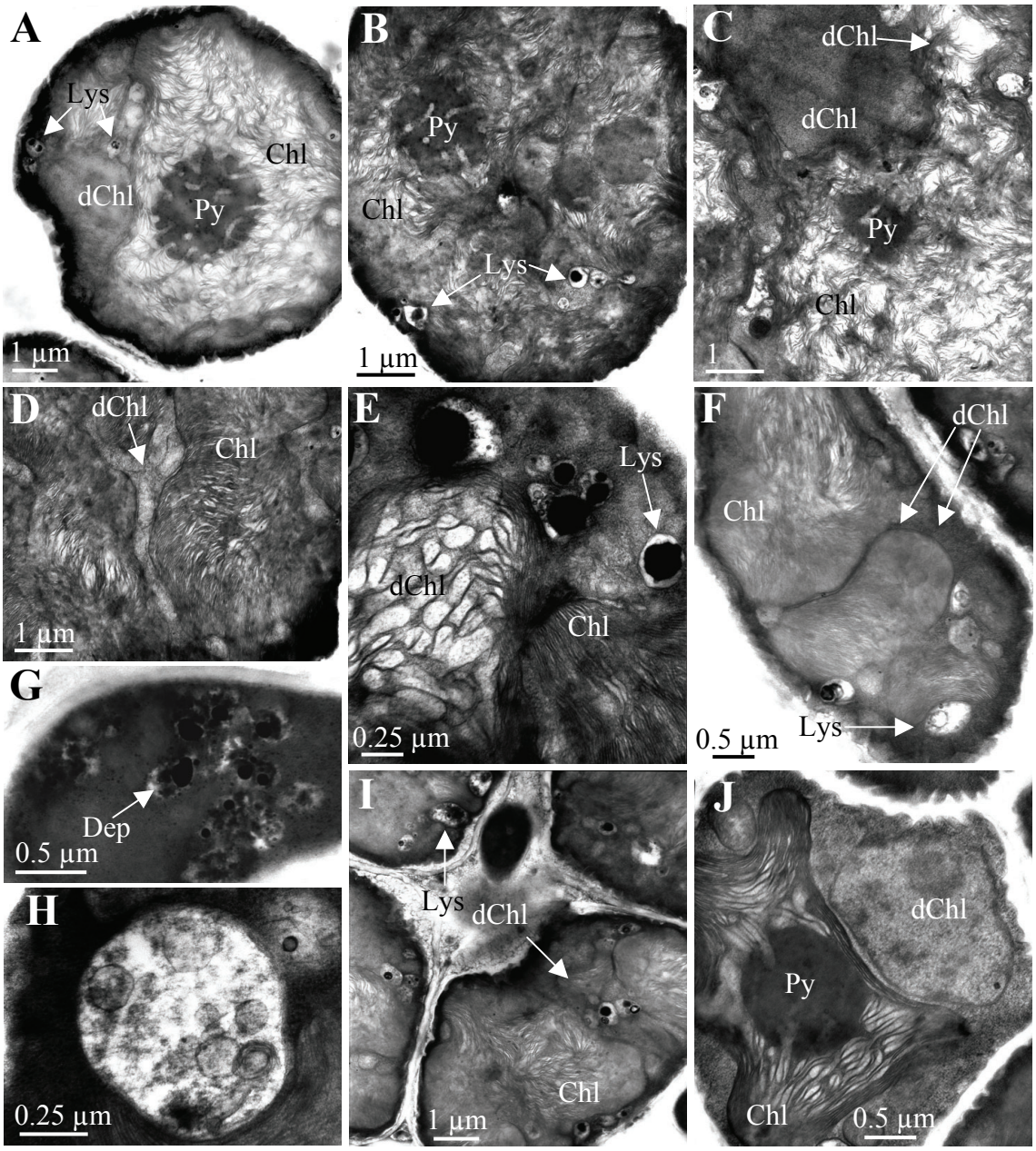

Fig. 4-2. TEM micrographs of ultrathin sections of soredia of *H. physodes* cultivated with 500 µM $MnCl_2$, showing grown (A, C, F, G, I) or not grown soredia (B, D, E, H, J). A, B. Vegetative photobiont cells with central pyrenoid (Py) and reduced chloroplast (Chl) with lysosome-like vesicles (Lys) in areas where the chloroplast is decomposed (dChl). C. Vegetative photobiont cell with damaged thylakoids and areas with completely decomposed chloroplast. D. Vegetative photobiont cell with partly intact and partly decomposed chloroplast. E. Contracted thylakoids in damaged parts of the chloroplast (dChl) in vegetative photobiont cell. F. Autospore of *T. jamesii* with partly decomposed chloroplast and lysosome-like vesicles in destroyed areas. G. Mycobiont hypha with electron-dense deposits (Dep) possibly containing Mn. H. Lysosome-like vesicle in vegetative photobiont cell. I. Photobiont autospores with partly decomposed chloroplast and several lysosome-like vesicles. J. Autospore with chloroplast strongly reduced in size and contracted thylakoids in the remaining parts.

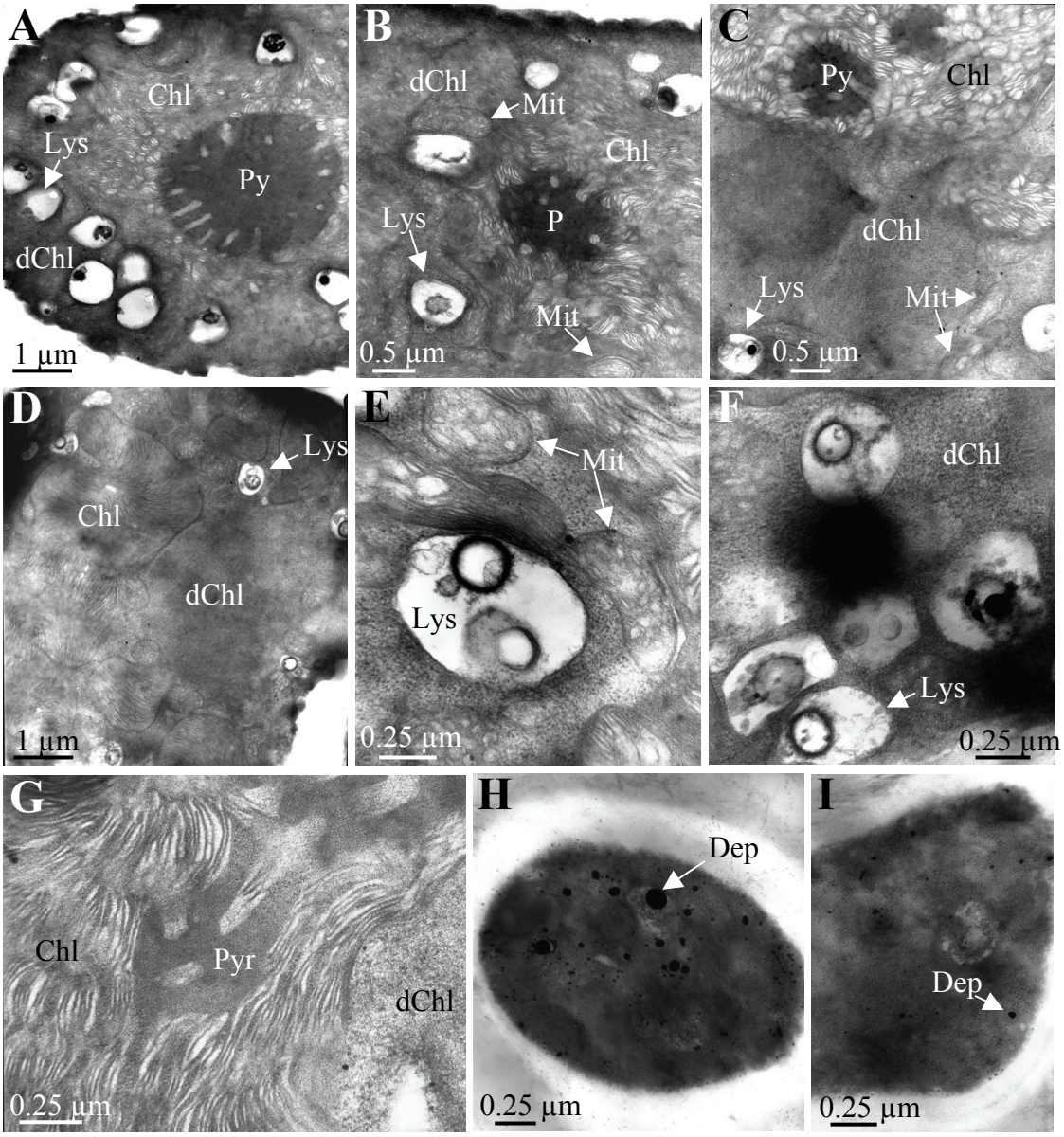

Fig. 4-3. TEM micrographs of ultrathin sections of soredia of *H. physodes* cultivated with 7 mM $MnCl_2$, showing grown (A - C, E, H) or not grown soredia (D, F, G, I). A. Vegetative photobiont cell with central pyrenoid (Py), strongly reduced chloroplast (Chl) and numerous lysosome-like vesicles (Lys) in areas where the chloroplast is decomposed (dChl). B. Lysosome-like vesicles in decomposed areas of the chloroplast occur in central parts of vegetative photobiont cell close to the pyrenoid; Mit: mitochondrion. C. Contracted thylakoids in strongly reduced chloroplast of vegetative photobiont cell. D. Vegetative photobiont cell with intact and decomposed areas of the chloroplast. E. Lysosome-like vesicle and mitochondria in vegetative photobiont cell. F. Vegetative photobiont cell with numerous lysosome-like vesicles. G. Chloroplast reduced in size and with contracted thylakoids in vegetative photobiont cell. H, I. Mycobiont hyphae with electrone-dense deposits (Dep).

4. Discussion

The damage of thylakoids and the disintegration of parts of the chloroplast at 500 µM Mn and higher is in line with reduced chlorophyll concentrations that were observed in Mn-exposed soredia and thallus pieces of *H. physodes* (HAUCK et al. 2002d, 2003). The chloroplast damage is probably due to the high affinity of thylakoid-bound ferredoxins for heavy metals (SABNIS et al. 1969, MCQUATTIE & SCHIER 2000). The structural damage in the chloroplast increased with increasing Mn concentration and was higher in not grown versus grown soredia at 500 µM Mn. Reduced chloroplasts were already found in TEM studies with semithin sections of *H. physodes* soredia cultivated with 7 mM Mn (HAUCK et al. 2002d). The high frequency of lysosome-like vesicles in areas formerly filled by the chloroplast is thought to be the result of the degradation of damaged parts of the chloroplast. An increase of lysosome-like vesicles in the cytoplasm concomitant to the degradation of the chloroplast was already found in ageing cells of *Myrmecia biatorellae* in *Catapyrenium* spec. (GALUN et al. 1971). Swelling of mitochondrial cristae in the photobiont was too rarely observed to attribute this to the varying Mn concentrations in the culture medium. TARHANEN (1998) found swollen cristae in the mitochondria of *Trebouxia jamesii* cells in *Bryoria fuscescens* subsequent to the exposure to mixed solutions of H_2SO_4, $CuSO_4$ and $NiSO_4$ at pH 3. Swelling of mitochondrial cristae and thylakoids is a non-specific symptom indicating initial stages of cell death caused by various kinds of environmental stress (SIEVERS 1966, TARHANEN 1998). Autospores of *T. jamesii* were apparently more susceptible to Mn than vegetative cells. While the chloroplasts were more severely damaged at 500 µM Mn in autospores versus vegetative cells, autospore formation was suppressed at 7 mM Mn suggesting that excess Mn affects the reproduction of the photobiont. The cause for the higher Mn sensitivity of the autospores could be the different cell wall ultrastructure of autospores and vegetative cells in *Trebouxia* growing in lichen thalli (HONEGGER 1984). Zoospores, which are regularly formed by *T. jamesii* in culture, but have been rarely observed in lichen thalli (SLOCUM et al. 1980, FRIEDL & BÜDEL 1996), were not found in the present study.

Ultrastructural changes in the mycobiont were the same at both Mn concentrations (500 µM or 7 mM) and in grown or not grown soredia. Electron-dense deposits in hyphae of Mn-treated soredia probably contained Mn, as the mycobiont of *H. physodes* is known

to accumulate Mn intracellularly in polyphosphate granules and other deposits (HAUCK et al. 2002d, PAUL et al. 2003). Mesosome-like structures are thought to be involved in the metabolite transfer between the two partners of the lichen symbiosis, especially by storing metabolites received from the photobiont in the mycobiont (PEVELING 1972, 1976, BOISSIÈRE 1982). Thus, their absence from all Mn-treated samples indicates that already relatively low Mn concentrations may severely impair the metabolite transfer to the mycobiont. In addition, HAUCK et al. (2002d) showed with SEM that the dense network of hyphae covering the photobiont in control samples was degenerated in *H. physodes* soredia cultivated with 7 mM Mn. Hence, Mn apparently injures both the structural and the metabolic interface between the fungus and the alga in the soredia. The cytoplasma tufts in the photobiont remained apparently unaffected by the Mn treatments. The tufts are assumed to promote the metabolite transfer, because they enlarge the algal surface at the interface to the mycobiont. GALUN et al. (1970) found such tufts in the photobiont of *Lecanora olea* and *L. rupicola* ssp. *subplanata*, as did PEVELING (1969) in the mycobiont of *Dermatocarpon miniatum*. The fungus/alga interface in *H. physodes* is formed by intraparietal haustoria (FIECHTER & HONEGGER 1988).

Concentric bodies are specific organelles of fungi, primarily of lichenized species (GRIFFITH & GREENWOOD 1972), and have been assumed to be involved in membrane repair (AHMADJIAN 1993), e.g., enabling the fungus efficiently to repair membranes after desiccation (GALUN et al. 1974, BOISSIÈRE 1982, PEVELING et al. 1985). This assumption is supported by the absence of concentric bodies from *Hydrothyria venosa*, a permanently submerged aquatic lichen (JACOBS & AHMADJIAN 1973). Their absence from soredia of *H. physodes* cultivated with Mn could be due either to a direct disintegration caused by Mn or they could be used up because of intense membrane repair activity. In either case, Mn-treated soredia can be assumed to lose their desiccation resistence due to the loss of concentric bodies. In their natural habitats, the soredia frequently underly drying and rewetting cycles (OTT 1987). HONEGGER (1995) and HONEGGER et al. (1996), however, speculated that concentric bodies could be remnants of gas bubbles that are frequent in desiccated cells of drought-resistant fungi. As long as the function of the concentric bodies is controversial, all interpretations to their absence in Mn-treated samples of the present experiments are preliminary. In addition to the possible reduction of the drought resistance by affecting the concentric bodies, Mn can be assumed to impair the water relations in *H. physodes* because of the reduced physical

contact between mycobiont and photobiont found by HAUCK et al. (2002d). This is relevant to the water supply of the photobiont, because HONEGGER et al. (1996) showed in thalli of *H. physodes* and other lichen species that free water is generally confined to the cell interior and to the cell walls, even in fully water-saturated thalli. This implies that the fungal apoplast is the only water source for the photobiont in stratified lichen thalli (HONEGGER et al. 1996); in intact heteromerous thalli, all photobiont cells are connected to the mycobiont (HONEGGER 1991).

At 7 mM $MnCl_2$, no significant difference was observed in the ultrastructure between grown and not grown soredia. Grown soredia also suffered ultrastructural damage from the exposure to Mn. This suggests that the establishment of soredia, which were capable of surviving the first days, might not necessarily be permanent. Since already the rate of soredia that successfully survive the first days at 7 mM $MnCl_2$ is small (25 % of the control after 8 days in HAUCK et al. 2002d), only very few soredia can be expected to develop thalli on substrates with high Mn concentrations. In a transplantation experiment with soredia of *H. physodes* on *Betula* bark near a copper smelter, MIKHAILOVA & SCHEIDEGGER (2001) found a significant decrease of living soredia even between the 12th and the 16th month after transplantation. On *Picea abies* in the Harz Mountains, *H. physodes* was completely absent from bark containing 5 mmol Mn kg^{-1} dry weigth or more (HAUCK 2000).

The ultrastructural changes observed in soredia of *H. physodes* cultured with 500 µM or 7 mM $MnCl_2$ provide further support for the hypothesis that high ambient Mn concentrations in bark and stemflow are capable of limiting epiphytic lichen abundance in conifer forests. There is multiple evidence now that excess Mn impairs energy gain and carbon acquisition of the lichen symbiosis by reducing chlorophyll concentrations and destroying chloroplasts. The present results also suggest that drought resistance, an important feature of lichen symbiosis, is reduced by Mn. However, as long as the function of concentric bodies has not been clarified, this has to remain speculation. The suppression of autospore formation suggests that Mn inhibit the reproduction of the photobiont. In conclusion, ultrastructural changes observed in the present study give new insights in mechanisms that could lead to Mn sensitivity of *H. physodes* and other epiphytic lichen species.

Chapter 5

Manganese uptake in the epiphytic lichens *Hypogymnia physodes* and *Lecanora conizaeoides*

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Abstract

Exposure of the foliose epiphytic lichen *Hypogymnia physodes* to 100 mM $MnCl_2$ led to rapid adsorption to extracellular cation exchange sites; saturation was reached within about 50 min. Intracellular uptake was constant in samples exposed to 100 mM $MnCl_2$ for 10 to 120 min and analyzed for their Mn concentration after one day of recovery in the growth chamber. About 15 times as much Mn was bound extracellularly than was taken up intracellularly. Both 50 mM $CaCl_2$ and 50 mM $MgCl_2$ applied together with 100 mM $MnCl_2$ significantly reduced Mn uptake intracellularly and binding in the extracellular exchange sites. $CaCl_2$ was more effective than $MgCl_2$ at reducing Mn uptake intracellularly; extracellularly $CaCl_2$ and $MgCl_2$ reduced Mn adsorption equally. In the assays with $MnCl_2$, alone or in combination with $CaCl_2$, the amount of extracellularly bound Mg decreased, as did the content of Ca in $MnCl_2$ alone or in combination with $MgCl_2$. The results support the hypothesis that the alleviating effects of Ca and Mg on Mn toxicity, documented in previous culture experiments with *H. physodes*, were, at least in part, due to reduced Mn uptake. *H. physodes* bound significantly more Mn from 1 - 100 mM $MnCl_2$ solutions on its extracellular exchange sites than did the crustose *Lecanora conizaeoides*. Further, *H. physodes*, but not *L. conizaeoides* released significant amounts of Ca and Mg from the extracellular exchange sites during Mn uptake. Intracellular Mn concentrations increased and the Ca/Mn and the Mg/Mn ratios decreased with increasing Mn supply in either species.

The intracellular Mg/Mn ratio was higher in *L. conizaeoides* than in *H. physodes*. Its lower extracellular Mn uptake combined with lower losses of Ca and Mg as well as the higher intracellular Mg/Mn ratio may contribute to higher Mn tolerance of *L. conizaeoides* in the field, which was observed in spruce forests of the Harz Mountains. Neither *H. physodes* nor *L. conizaeoides* had significant amounts of extracellularly bound Mn^{3+} or Mn^{4+} when compared to the concentration of Mn^{2+}. Thus, immobilization of Mn^{2+} by oxidizing, as known from non-lichenized green algae, is unlikely as tolerance mechanism in *L. conizaeoides*.

1. Introduction

HAUCK et al. (2002) showed in culture experiments with agar media that growth of soredia of the epiphytic lichen *Hypogymnia physodes* is inhibited by ambient Mn concentrations. These results agree with the negative correlations found between the cover of *H. physodes* and the Mn content in *Picea abies* bark in the Harz Mountains, northern Germany (HAUCK 2000; HAUCK et al. 2001b). As soredia are the most important diaspores of *H. physodes* for short-distance dispersal (ARMSTRONG 1987), a correlation can be expected between cover values and the ability of the soredia to grow. The Mn content of *H. physodes* in the Harz Mountains depends on the Mn content of the colonized spruce bark (HAUCK 2000). The bark is supplied with Mn taken up by the root system and translocated through the xylem (LÖVESTAM et al. 1990). Thus, the Mn content of the soil, varying naturally as well as due to anthropogeneous soil acidification, has an indirect impact on the performance of *H. physodes*.

In the culture experiments with *H. physodes* soredia, Ca and Mg compensated for the Mn-induced growth depression (HAUCK et al. 2002d). Moreover, cover of *H. physodes* was found to decrease with decreasing Ca/Mn ratio of stemflow. At two different sites in the Harz Mountains, cover exceeded 1 % only if the mean molar ratio of Ca/Mn in stemflow exceeded 20. In the present study, we tested, therefore, the hypothesis that Ca and Mg reduce Mn uptake in *H. physodes*.

Our field studies in the Harz Mountains also revealed that cover of the crustose lichen *Lecanora conizaeoides* was not affected by the Mn content in bark or stemflow (HAUCK 2000). Furthermore, unpublished results of our group show that short-term exposure to $MnCl_2$ solutions reduced the chlorophyll content of *H. physodes*, but did not affect the chlorophyll content of *L. conizaeoides*, even at higher Mn concentrations than applied to *H. physodes*. Therefore, we conducted comparative experiments in order to test the hypothesis that *L. conizaeoides* takes up less Mn from the same source than *H. physodes*. KNAUER et al. (1999) found that the green alga *Scenedesmus subspicatus* immobilizes Mn extracellularly by oxidizing Mn^{2+} to Mn^{3+} and Mn^{4+}. By applying the extraction procedure of KNAUER et al. (1999) to *H. physodes* and *L. conizaeoides*, we further tested the hypothesis that the lower Mn sensitivity of *L. conizaeoides* is due to its ability to oxidize Mn^{2+} to Mn^{3+} and Mn^{4+}. Results of these experiments are presented below.

2. Materials and methods

2.1. Time-dependent Mn uptake in *Hypogymnia physodes* in competition with Ca and Mg

Hypogymnia physodes (L.) Nyl. was collected in the Göttingen area and stored in a plastic bag in dark at 5 - 10 °C for at least two days. The moist thalli were cut into pieces of about 1 cm^2 (c. 30 mg d. wt.). These pieces were mixed in order to avoid effects due to different vitality of individual thalli and stored in Petri dishes for one day at 80 % relative humidity, a day temperature (for 13 hours daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1}, and a night temperature of 10 °C. Twenty pieces per replicate were incubated in 20 ml either of (1) deionized water, (2) 100 mM $MnCl_2$, (3) 100 mM $MnCl_2$ and 50 mM $CaCl_2$, or (4) 100 mM $MnCl_2$ and 50 mM $MgCl_2$ for 0, 10, 20, 30, 40, 50 and 60 min. In the assay with 100 mM $MnCl_2$, lichen pieces were also incubated for 120 min. Five replicates samples after incubation were briefly washed in 20 ml of deionized water and stored in a growth chamber under climatic conditions as described above for one day. With this day in the growth chamber prior to analysis, uptake or release processes that do not take place immediately during Mn exposure should be included. This procedure differs from those of other metal uptake studies,

where samples were analyzed directly after incubation (NIEBOER et al. 1978). It was choosen for all experiments of the present study, as it comes closer to field conditions. During precipitation events epiphytic lichens are temporarily exposed to elevated Mn concentrations (HAUCK 2000, LEVIA & HERWITZ 2000), but have more or less extended recovery periods afterwards. Cations trapped in intercellular spaces were eluated by shaking the samples twice for 20 min with 20 ml of deionized water; next, ions bound extracellularly in anionic exchange sites were extracted by shaking twice for 20 min with 20 ml of 20 mM $NiCl_2$. Afterwards, samples were dried at 105 °C, homogenized, and digested with 65 % HNO_3 in order to determine the intracellular ions (BROWN & BROWN 1991). According to VÁZQUEZ et al. (1999) two washing procedures with 20 mM $NiCl_2$ are sufficient to release extracellularly bound ions of class A metals or borderline ions with class A character (NIEBOER & RICHARDSON 1980), whereas $NiCl_2$ incubation with higher concentrations or for prolonged periods results in membrane damage. Concentrations of extracellularly bound and of intracellular Mn, Ca, Mg and K (the latter for assessing membrane integrity) were measured separately in each of the $NiCl_2$ solutions and of the acidic digests with AAS (Varian, SpectrAA 30). Ca was measured with K^+ as a deionizer, as was Mg with La^{3+} and K with Cs^+. Element concentrations were related to the dry weight of the lichen samples. Amounts bound in extracellular exchange sites were calculated by adding the results from the two $NiCl_2$ extractions per replicate.

2.2. Concentration-dependent Mn uptake in *Hypogymnia physodes* and *Lecanora conizaeoides*

Pieces of *H. physodes* were prepared and stored as described in 2.1. Thalli of the crustose *Lecanora conizaeoides* Nyl. ex Crombie were also sampled in the Göttingen area and scraped off some boles with a razor blade. For incubation in the growth chamber, the very small thallus fragments of *L. conizaeoides* (c. 10 mg d. wt.) were evenly spread on the surface of filter papers in Petri dishes. They were removed by filtering from the incubation medium and the extractants, whereas the foliose *H. physodes* could be removed from the solutions with forceps after decantation. After preincubation for one day in the growth chamber, samples were incubated in five replicates with 20 ml of 0, 1 and 100 mM $MnCl_2$ for 1 h. *L. conizaeoides* was additionally exposed to 10 mM $MnCl_2$. Subsequent to incubation, samples were shortly washed with

deionized water and stored for one day in the growth chamber. Using the procedure of KNAUER et al. (1999), they were shaken for 20 min in 20 ml each, sequentially, of (1) deionized water, (2) 20 mM Na_2-EDTA, (3) 20 mM ascorbate, and (4) 20 mM Na_2-EDTA. Steps (1) and (4) were repeated twice, and step (2) four times. The number of repetitions required for the quantitative Mn^{2+} extraction with EDTA was determined in a preliminary test. Water eluted the free apoplastic ions, whereas the subsequent EDTA treatments extracted extracellularly bound Mn^{2+}. Ascorbate as a mild reducing agent converted extracellularly bound Mn^{3+} and Mn^{4+} into Mn^{2+}. Thus, the Mn content of the ascorbate solution and of the EDTA solution applied afterwards reflects the content of Mn^{3+} and Mn^{4+}. EDTA had the same function as $NiCl_2$ in the first experiment; the different extractants were used in order to follow the standard procedures of BROWN & BROWN (1991) for the first experiment and of KNAUER et al. (1999) for the second. As Mn^{2+} is a borderline ion with strong class A character (NIEBOER & RICHARDSON 1980), any significant difference in the efficiency of the two extractants is not be expected (VÁZQUEZ et al. 1999). The intracellular fraction was obtained by acid digestion. Concentrations of Mn, Ca and Mg were measured with AAS in the EDTA and ascorbate extracts as well as in the acid digests. Data given in parts 3.2 and 3.3 of the results section are based on the same experiment.

2.3. Statistics

Arithmetic means ± standard error (SE) are given throughout the paper. As all data were normally distributed (Shapiro-Wilk test), Student's *t*-test was employed for determining significant differences between samples. An analysis of variance (ANOVA) was carried out in order to quantify to what extent the intra- and extra-cellular Mn concentrations in *H. physodes* were due to the incubation medium or to the duration of incubation (Table 5-1). Pairs of different incubation media were compared in the analysis for detecting significant differences between the treatments with $MnCl_2$ alone and in combination with $CaCl_2$ or $MgCl_2$. Only samples incubated for 10 - 60 min were included in the ANOVA, as the rapid change of Mn contents of *H. physodes* during the first 10 min blurred the effect of Mn concentrations in the incubation media. Statistical significance was tested by calculating *F* values (BORTZ 1999). Statistical analyses were computed with SAS 6.04 software (GOGOLOK et al. 1992). Michaelis-Menten regression lines for

intra- and extra-cellular Mn uptake in Figs. 5-1 and 5-2 were calculated with the program Xact 4.01, SciLab Co., Hamburg.

3. Results

3.1. Effects of Ca and Mg on Mn uptake in *Hypogymnia physodes*

When *H. physodes* was exposed to 100 mM $MnCl_2$, intracellular Mn concentration amounted to about 7.5 µmol g^{-1} d. wt. irrespective of the duration of the treatment between 10 and 120 min (Fig. 5-1). Further, 10 min of incubation were sufficient to achieve a saturation of intracellular Mn concentration, when 100 mM $MnCl_2$ was applied together with 50 mM $CaCl_2$ or $MgCl_2$, but the saturation point was lower, i.e., at about 5 µmol g^{-1} d. wt. ANOVA showed that this difference was statistically significant (Table 5-1). $CaCl_2$ was more effective in inhibiting intracellular Mn uptake than $MgCl_2$ (Table 5-1), even though the difference was small. The intracellular Mn concentration of the samples treated for 10 to 60 min was 4.75 ± 0.18 µmol g^{-1} d. wt. in the assay with $CaCl_2$ and 5.30 ± 0.18 µmol g^{-1} d. wt. in that with $MgCl_2$. Extracellular Mn uptake was about 15 times as high as intracellular uptake (Fig. 5-2); it was not completed in the variants exposed to Mn for less than about 50 min.

Table 5-1. Effects of treatment quality and duration on the intracellular and extracellularly bound Mn content of *Hypogymnia physodes*.

Contrast between	Total		Medium		Time[c]		Medium × time	
treatments	Var. [%]	F value[b]	Var. [%]	F value	Var. [%]	F value	Var. [%]	F value
Intracellular:								
Mn; Ca[a]	87	28.76***	71	280.8***	13	8.33***	4	2.85*
Mn; Mg	79	16.49***	59	147.7***	12	5.02***	8	4.02**
Ca; Mg	47	3.83**	15	13.54***	22	3.99**	9	1.72
Mn; H_2O	96	94.33***	90	800.3***	5	7.59***	0	1.57
Ca; H_2O	96	118.4***	93	913.3***	2	4.82**	1	4.99*
Mg; H_2O	94	65.75***	91	512.3***	2	2.22	0	1.27
Extracellular:								
Mn; Ca	65	7.98***	15	21.50***	33	8.23***	17	4.99***
Mn; Mg	38	2.72**	22	19.10***	10	1.46	6	0.98
Ca; Mg	58	6.03***	0	0.07	36	8.26***	22	5.00***
Mn; H_2O	89	34.69***	78	295.6***	4	2.37*	7	1.16
Ca; H_2O	93	62.97***	68	364.4***	25	27.52***	0	0.88
Mg; H_2O	84	23.48***	83	185.2***	1	0.50	0	0.03

[a] Assays included in the analysis: Mn — 100 mM $MnCl_2$, Ca — 100 mM $MnCl_2$ + 50 mM $CaCl_2$, Mg — 100 mM $MnCl_2$ + 50 mM $MgCl_2$, H_2O — control with deionized water.
[b] Levels of significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (ANOVA; $n = 5$).
[c] Only samples incubated for 10 - 60 min considered.

However, the increase in extracellular Mn concentrations between 10 and 120 min was statistically insignificant. $CaCl_2$ and $MgCl_2$ reduced the extracellular Mn uptake equally and statistically significantly (Table 5-1). However, ANOVA revealed a considerably higher effect of $CaCl_2$ and $MgCl_2$ on intracellular than on extracellular Mn uptake.

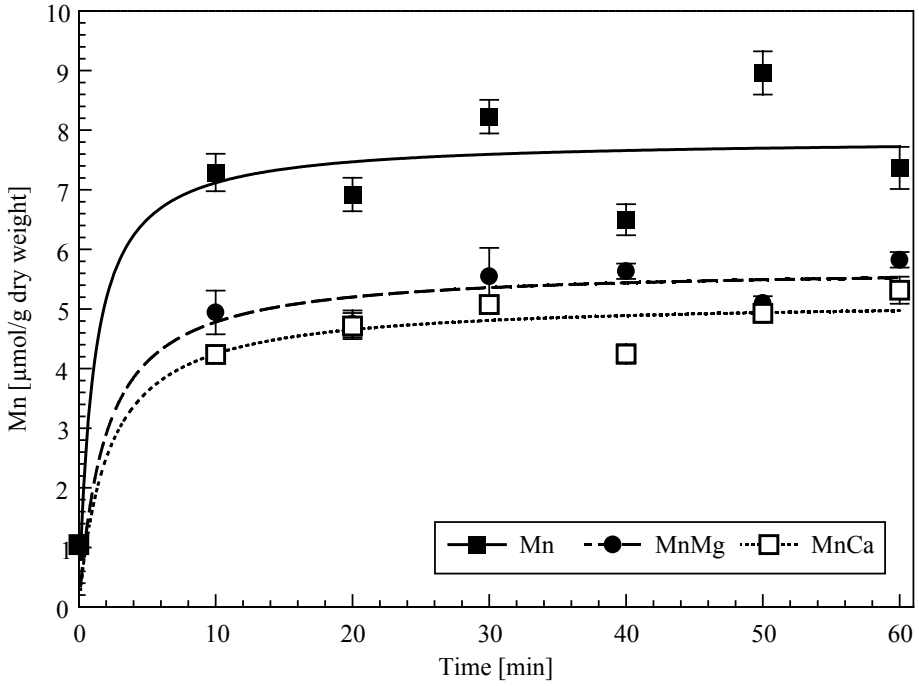

Figure 5-1. Intracellular Mn uptake in *Hypogymnia physodes* related to time from 100 mM $MnCl_2$ solution alone or combined with 50 mM $CaCl_2$ or $MgCl_2$. After 120 min (not shown) 8.9 ± 0.6 µmol Mn per g dry weight were taken up from 100 mM $MnCl_2$ solution. Arithmetic means of five replicates ± SE.

Mn uptake did not alter the intracellular content of Ca, Mg and K, regardless whether $MnCl_2$ was applied alone or together with $CaCl_2$ or $MgCl_2$ (Tables 5-2 – 5-4). Extracellularly bound Mg was significantly reduced by the treatments with $MnCl_2$ alone or with $MnCl_2$ and $CaCl_2$. In the assay with $MnCl_2$ and $MgCl_2$ in combination, it remained constant in the variants treated for up to 40 min, but increased afterwards, though this increase was statistically insignificant due to high variation between the replicates. Concentration of extracellularly-bound Ca decreased in the treatments with $MnCl_2$ and with $MnCl_2$ plus $MgCl_2$, but was not altered in the treatment with $MnCl_2$ and $CaCl_2$. The concentration of extracellularly bound K was considerably lower than that of Ca and Mg. In some variants it was lower than in the control sample, however, without exhibiting a trend to decreasing concentrations with increasing Mn supply. Concentrations of all cations showed more variation in the extracellular bindings sites than in the intracellular fraction.

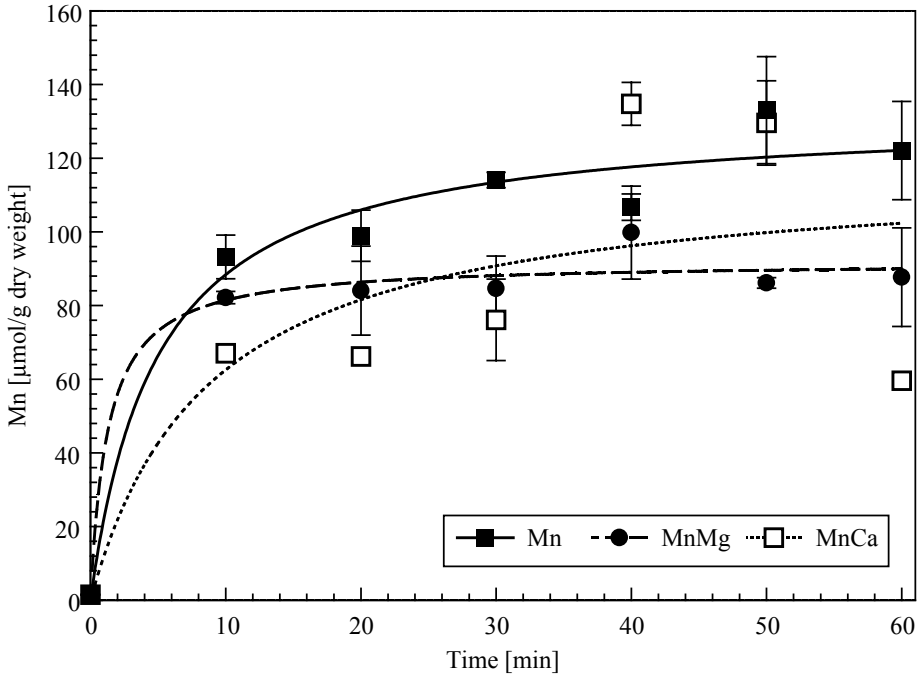

Figure 5-2. Mn uptake in extracellular exchange sites of *Hypogymnia physodes* related to time from 100 mM $MnCl_2$ solution alone or combined with 50 mM $CaCl_2$ or $MgCl_2$. After 120 min (not shown) 133 ± 15 µmol Mn per g dry weight were taken up from 100 mM $MnCl_2$ solution. Arithmetic means of five replicates ± SE.

3.2. Mn uptake in *Hypogymnia physodes* and *Lecanora conizaeoides*

Before incubation the content both of intracellular and of extracellularly bound Mn was the same in *H. physodes* and in *L. conizaeoides*. Intra- and extra-cellular Mn content increased in both lichen species with increasing $MnCl_2$ content of the incubation medium (Table 5-5). At the extracellular cation exchange sites, Mn occurred in significantly higher concentrations in *H. physodes* than in *L. conizaeoides*. Related to dry weight, *H. physodes* bound as much Mn from 1 mM $MnCl_2$ extracellularly as did *L. conizaeoides* from 100 mM $MnCl_2$ solution. The intracellular Mn content did not differ between the species. $MnCl_2$ reduced the content of extracellularly bound Ca and Mg in *H. physodes*, but not in *L. conizaeoides*, although concentrations did not differ between the lichen species before the experiment. Similarly, increasing $MnCl_2$ exposure caused a much higher increase in the Ca and in the Mg content of the incubation medium with *H. physodes* than with *L. conizaeoides*. Intracellular concentrations of Ca and Mg were not affected by Mn uptake. $MnCl_2$ reduced the Ca/Mn and Mg/Mn ratios in both lichen

species; however, this trend was statistically insignificant for the ratio of intracellular Ca/Mn in *L. conizaeoides*. Extracellular Ca/Mn and Mg/Mn ratios did not differ between the controls of both lichen species, but decreased stronger in *H. physodes* than in *L. conizaeoides*. The intracellular Mg/Mn ratio was higher in *L. conizaeoides* than in *H. physodes*, whereas it was the other way round with the intracellular Ca/Mn ratio.

Table 5-2. Intracellular and extracellularly bound Ca concentrations in *Hypogymnia physodes* (in µmol g^{-1} d. wt.) after treatment with 100 mM $MnCl_2$ alone or in combination with 50 mM $CaCl_2$ or 50 mM $MgCl_2$.[a]

Duration [min]	$MnCl_2$		$MnCl_2 + CaCl_2$		$MnCl_2 + MgCl_2$	
Intracellular:						
0	786 ± 23	ab	786 ± 23	a	786 ± 23	a
10	737 ± 31	abc	544 ± 29	b	663 ± 65	ab
20	831 ± 34	a	508 ± 45	bc	482 ± 43	bc
30	669 ± 42	bc	421 ± 23	c	477 ± 24	bc
40	817 ± 66	abc	932 ± 32	d	675 ± 80	abc
50	795 ± 42	ab	747 ± 52	a	808 ± 59	a
60	777 ± 36	ab	488 ± 70	bc	467 ± 17	c
120	652 ± 31	c	.		.	
Extracellular:						
0	54.6 ± 3.7	a	54.6 ± 3.7	abc	54.6 ± 3.7	a
10	38.5 ± 4.2	bd	46.9 ± 1.4	b	32.8 ± 2.1	b
20	21.5 ± 5.0	c	52.2 ± 3.8	ab	20.4 ± 1.4	c
30	28.8 ± 5.1	bc	63.8 ± 12.1	abcd	24.8 ± 2.2	c
40	53.2 ± 5.0	ad	71.6 ± 5.5	cd	35.9 ± 2.8	b
50	37.9 ± 3.0	bd	63.9 ± 4.3	d	37.5 ± 3.4	b
60	33.3 ± 4.7	bc	45.7 ± 2.0	ab	22.4 ± 2.4	c
120	33.1 ± 2.5	bc	.		.	

[a] Arithmetic mean ± SE. Statistics: *t*-test, $P \leq 0.05$, df = 4. Within a column, separately for intracellular and extracellularly bound concentrations, means sharing a common letter do not differ significantly.

3.3. Exchangeable extracellular Mn^{2+} vs. Mn^{3+} and Mn^{4+} in *Hypogymnia physodes* and *Lecanora conizaeoides*

Mn concentrations in the ascorbate fraction and the two subsequent EDTA fractions (Mn^{3+}, Mn^{4+}) were negligible in comparison to those in the first four EDTA fractions (Mn^{2+}). *H. physodes* had 150-200 times and *L. conizaeoides* 30-40 times higher concentrations of extracellularly bound Mn^{2+} than of Mn^{3+} and Mn^{4+} (Table 5-6). In both lichen species and both fractions, Mn content increased with increasing $MnCl_2$ content of the incubation medium.

Table 5-3. Intracellular and extracellularly bound Mg concentrations in *Hypogymnia physodes* (in µmol g^{-1} d. wt.) after treatment with 100 mM $MnCl_2$ alone or in combination with 50 mM $CaCl_2$ or 50 mM $MgCl_2$.[a]

Duration [min]	$MnCl_2$		$MnCl_2 + CaCl_2$		$MnCl_2 + MgCl_2$	
Intracellular:						
0	15.3 ± 0.6	ab	15.3 ± 0.6	ab	15.3 ± 0.6	abc
10	14.2 ± 0.9	ab	15.2 ± 0.5	a	15.1 ± 0.3	ac
20	13.2 ± 0.6	a	15.2 ± 0.9	ab	16.4 ± 0.5	ac
30	17.9 ± 2.7	ab	14.9 ± 0.4	ab	17.1 ± 0.7	a
40	14.5 ± 0.4	ab	13.4 ± 0.5	b	13.5 ± 0.3	b
50	15.1 ± 0.4	b	14.5 ± 1.0	ab	14.6 ± 0.5	bc
60	14.0 ± 0.4	ab	16.9 ± 0.9	a	16.2 ± 0.8	ac
120	15.0 ± 0.8	ab	.		.	
Extracellular:						
0	31.1 ± 3.4	a	31.1 ± 3.4	a	31.1 ± 3.4	ab
10	5.44 ± 0.78	b	1.85 ± 0.19	b	27.2 ± 0.8	a
20	2.69 ± 0.54	c	1.47 ± 0.18	b	25.3 ± 0.5	ab
30	2.13 ± 0.39	c	1.94 ± 0.20	b	24.5 ± 0.7	b
40	6.73 ± 0.65	b	1.95 ± 0.14	b	26.9 ± 0.8	ab
50	4.52 ± 0.39	bd	1.98 ± 0.12	b	58.1 ± 17.9	ab
60	2.62 ± 0.63	cd	2.36 ± 0.36	b	56.1 ± 16.8	ab
120	2.65 ± 0.42	c	.		.	

[a] Arithmetic mean ± SE. Statistics: *t*-test, $P \leq 0.05$, df = 4. Within a column, separately for intracellular and extracellularly bound concentrations, means sharing a common letter do not differ significantly.

4. Discussion

The experiments showed a reduced Mn uptake in *H. physodes* in the presence of Ca and Mg. Thus, the compensating effect of Ca and Mg observed for Mn-induced growth depression in *H. physodes* soredia (HAUCK et al. 2002d) can, at least in part, be attributed to reduced intra- and extra-cellular Mn uptake. Further, the results yield an explanation of the correlation between cover of *H. physodes* and the Ca/Mn ratio of stemflow as found in the Harz Mountains. Mn uptake was already proven to be reduced by Ca in the green alga *Kirchneriella lunaris* (ISSA et al. 1995) and by Mg in the yeast *Saccharomyces cerevisiae* (BLACKWELL et al. 1998). Competition for extracellular binding sites such as carboxyl groups is thought to be the cause for the reduced uptake (BROWN & BROWN 1991). Reduced Mn concentrations in the apoplast also led to lower intracellular uptake (RICHARDSON, 1995). We have no information whether the alleviating effect of Ca and Mg on Mn toxicity in *H. physodes* (HAUCK et al. 2002d) is solely caused by the lower Mn uptake or, additionally, by direct promotional effects of Ca and Mg.

Table 5-4. Intracellular and extracellularly bound K concentrations in *Hypogymnia physodes* (in µmol g^{-1} d. wt.) after treatment with 100 mM $MnCl_2$ alone or in combination with 50 mM $CaCl_2$ or 50 mM $MgCl_2$.[a]

Duration [min]	$MnCl_2$		$MnCl_2 + CaCl_2$		$MnCl_2 + MgCl_2$	
Intracellular:						
0	63.8 ± 4.6	abc	63.8 ± 4.6	ac	63.8 ± 4.6	abc
10	58.7 ± 4.7	abc	74.7 ± 2.8	a	71.0 ± 2.8	ac
20	53.2 ± 4.9	ac	77.9 ± 4.8	a	70.4 ± 4.1	ac
30	71.6 ± 4.6	b	68.2 ± 3.7	a	74.4 ± 3.9	a
40	54.2 ± 2.8	c	48.2 ± 3.7	b	52.1 ± 2.3	b
50	62.2 ± 0.6	ab	51.6 ± 4.2	bc	57.2 ± 4.9	bc
60	63.0 ± 4.5	abc	74.7 ± 5.6	a	76.8 ± 1.6	a
120	62.9 ± 3.4	abc	.		.	
Extracellular:						
0	2.11 ± 0.41	a	2.11 ± 0.41	a	2.11 ± 0.41	a
10	0.81 ± 0.13	b	0.54 ± 0.02	b	0.89 ± 0.13	bc
20	0.78 ± 0.15	b	1.31 ± 0.50	abc	1.53 ± 0.38	abc
30	2.19 ± 1.41	ab	1.10 ± 0.32	abc	1.70 ± 0.34	ab
40	2.03 ± 0.92	ab	0.57 ± 0.05	b	0.61 ± 0.04	c
50	2.19 ± 1.22	ab	0.89 ± 0.08	c	0.62 ± 0.09	c
60	3.48 ± 2.30	ab	0.92 ± 0.11	c	2.08 ± 0.78	a
120	1.01 ± 0.16	ab	.		.	

[a] Arithmetic mean ± SE. Statistics: t-test, $P \leq 0.05$, df = 4. Within a column, separately for intracellular and extracellularly bound concentrations, means sharing a common letter do not differ significantly.

The greater variation of the Mn concentration in the extracellular exchange sites than in the cellular content reflects the adjustment of an equilibrium in the former case and probably active uptake in the latter (NIEBOER et al. 1976, NASH 1989). Intracellular Mn uptake was probably completed subsequent to the incubation with the metal solution during the day of recovery in the growth chamber. In experiments where element concentrations were measured immediately after samples were treated with metal solution, it took particularly longer than 10 min for the intracellular concentration of the respective element to become satured. Intracellular uptake from 16 mM $CuSO_4$ solution, for instance, was saturated after about 90 min in *Usnea* spec. and after 60 min in *Ramalina fastigiata*, whereas excellular adsorption reached an equilibrium after 20 min in *Usnea* spec. and after 10 min in *R. fastigiata* (BRANQUINHO et al. 1997a). The extracellular binding sites are known to serve as a buffer between the cell interior and the environment. Cations adsorbed at the cell wall can be transferred into the cells later

on (BROWN 1991). In the field, Mn was proven to occur in marked peak concentrations in stemflow during winter (HAUCK 2000, LEVIA & HERWITZ 2000). The present results suggest that even short precipitation events could be sufficient to increase the intracellular Mn concentration in *H. physodes* by subsequent uptake from the extracellular exchange sites.

Mn uptake did not alter the intracellular K content indicating that, within the concentration range studied, loss of membrane integrity is not the cause for the sensitivity to Mn in *H. physodes*. This agrees with BURTON et al. (1981), who did not find a significant loss of K from *Cladonia rangiferina* following a 3-hour incubation with $MnCl_2$ at concentrations below 1 M. The membranes of cyanolichens, however, are apparently more susceptible to Mn (GOYAL & SEAWARD 1982). The intracellular K concentration of our control samples (64 ± 5 µmol g^{-1} d. wt.) is in the range of 40 — 140 µmol g^{-1} d. wt. found in *H. physodes* by BUCK & BROWN (1979) and WERNER (1993). Further, the intracellular Mg concentration remained unaffected by Mn, which also indicates that the membranes were intact (BRANQUINHO et al. 1997a). BRANQUINHO & BROWN (1994) found uptake of 0.1 mM $Pb(NO_3)_2$ in *Cladonia portentosa* to rapidly decrease extracellular concentrations of Ca and Mg within 1 h, whereas concentrations of intracellular Ca and Mg as well as of extracellular K remained relatively unchanged. These results parallel results found with Mn uptake in the present study. Intra- and extra-cellular Mn concentrations in *H. physodes* were generally higher in the controls and in samples treated for 1 h with 100 mM $MnCl_2$ in the first experiment presented in 3.1 (Figs. 5-1 – 5-2) than in the second experiment presented in 3.2 (Table 5-5).

Table 5-5. Intracellular and extracellularly bound Mn, Ca and Mg concentrations in *Hypogymnia physodes* and *Lecanora conizaeoides* (in µmol g^{-1} d. wt.) after treatment with 0 - 100 mM $MnCl_2$ for 1 h.[a]

	Hypogymnia physodes			*Lecanora conizaeoides*			
$MnCl_2$	0 mM	1 mM	100 mM	0 mM	1 mM	10 mM	100 mM
Intracellular:							
Mn	0.64 ± 0.02 a	0.67 ± 0.02 a	1.55 ± 0.12 b	0.72 ± 0.16 a	0.76 ± 0.06 ac	1.25 ± 0.26 ab	1.32 ± 0.12 bc
Ca	151 ± 8 a	145 ± 12 a	169 ± 14 a	6.10 ± 1.44 b	5.82 ± 0.62 b	4.78 ± 1.25 b	2.57 ± 0.38 b
Mg	2.73 ± 0.13 a	2.73 ± 0.18 a	2.52 ± 0.11 a	6.76 ± 0.63 b	7.15 ± 0.34 b	7.62 ± 0.78 b	5.85 ± 0.53 b
Ca/Mn	240 ± 16 a	216 ± 19 ab	113 ± 13 c	10.1 ± 3.1 de	8.18 ± 1.52 d	4.07 ± 1.00 de	2.09 ± 0.45 e
Mg/Mn	4.29 ± 0.11 a	4.07 ± 0.67 a	1.66 ± 0.21 b	10.8 ± 3.7 c	9.67 ± 1.61 c	6.72 ± 1.79 cd	4.50 ± 0.79 ad
Extracellular:							
Mn	1.63 ± 0.11 a	32.7 ± 1.6 b	97.5 ± 2.4 c	1.33 ± 0.31 a	10.2 ± 1.0 d	18.4 ± 1.2 e	28.2 ± 3.4 b
Ca	146 ± 7 a	120 ± 3 b	89.8 ± 2.2 c	121 ± 12 abd	138 ± 2 a	130 ± 23 abd	103 ± 7 cd
Mg	32.7 ± 1.6 a	23.8 ± 1.2 b	12.1 ± 0.3 c	36.6 ± 3.6 ad	43.1 ± 1.1 d	44.5 ± 10.2 abd	34.0 ± 2.5 a
Ca/Mn	90.3 ± 5.8 a	3.72 ± 0.16 b	0.92 ± 0.03 c	116 ± 25 a	14.0 ± 1.2 d	7.14 ± 2.26 e	3.83 ± 0.94 b
Mg/Mn	20.1 ± 4.0 a	0.73 ± 0.03 b	0.12 ± 0.00 c	35.4 ± 7.8 a	4.39 ± 0.34 d	2.43 ± 0.44 e	1.26 ± 0.13 e
Incubation medium:							
Ca	1.28 ± 0.13 a	12.6 ± 0.4 b	44.3 ± 1.7 c	1.30 ± 0.15 a	9.01 ± 0.83 d	10.7 ± 2.3 bd	15.3 ± 1.0 b
Mg	0.41 ± 0.05 a	10.9 ± 0.52 b	17.7 ± 1.7 c	0.39 ± 0.05 a	2.92 ± 0.39 d	3.32 ± 0.49 d	4.49 ± 0.80 d

[a] Arithmetic mean ± SE. Statistics: *t*-test, $P \leq 0.05$, df = 4. Within a row, means sharing a common letter do not differ significantly.

This is consistent with findings of BUCK & BROWN (1979) and BROWN (1991) that element concentrations in lichens from the same site underlie considerable temporal variation due to meteorological changes. The lichens for each experiment were collected on different days in order to use equally fresh thalli for each part of the study.

Table 5-6. Extracellularly bound Mn^{2+} vs. Mn^{3+} and Mn^{4+} in *Hypogymnia physodes* and *Lecanora conizaeoides* (in µmol g^{-1} d. wt.) after treatment with 0 - 100 mM $MnCl_2$ for 1 h.[a]

Species	$MnCl_2$	Mn^{2+}		Mn^{3+}/Mn^{4+}	
H. physodes	0	1.51 ± 0.09	a	0.10 ± 0.01	a
H. physodes	1	35.5 ± 1.4	b	0.15 ± 0.01	b
H. physodes	100	96.8 ± 2.1	c	0.65 ± 0.06	c
L. conizaeoides	0	1.14 ± 0.28	a	0.18 ± 0.01	b
L. conizaeoides	1	9.84 ± 0.84	d	0.31 ± 0.02	d
L. conizaeoides	10	17.9 ± 1.04	e	0.48 ± 0.06	cd
L. conizaeoides	100	27.3 ± 3.0	b	0.86 ± 0.24	cd

[a] Arithmetic mean ± SE. Statistics: *t*-test, $P \leq 0.05$, df = 4. Within a column, means sharing a common letter do not differ significantly.

The lower sensitivity of *L. conizaeoides* than of *H. physodes* to Mn cannot be explained by differences in intracellular uptake. Further, *L. conizaeoides* is not capable of converting Mn^{2+} into insoluble Mn^{3+} and Mn^{4+} in notable amounts. Even though the concentration of extracellularly bound Mn^{3+} and Mn^{4+} was significantly higher in *L. conizaeoides* than in *H. physodes*, it was still much lower than that of Mn^{2+}, and so Mn oxidation can be ruled out as the major cause for the different sensitivity of the two lichen species. In the green alga *Scenedesmus subspicatus*, KNAUER et al. (1999) found more than 90 % of the total Mn to be extracellularly bound as Mn^{3+} and Mn^{4+}. The low concentrations of Mn^{3+} and Mn^{4+} in the lichens suggest that no enzymes catalyzed the oxidation of Mn^{2+}. The higher extracellular uptake of Mn as well as the higher release of Ca and Mg from the apoplast in *H. physodes*, however, could be a cause for the higher sensitivity of this species to Mn. The lower Mg/Mn ratios in *H. physodes* could be another reason for the higher sensitivity. GOSS & CARVALHO (1992) and BLACKWELL et al. (1997) found the Mg/Mn ratio rather than the absolute Mn concentration to affect growth of wheat and yeast.

Chapter 6

Effects of manganese on element distribution and structure in thalli of the epiphytic lichens *Hypogymnia physodes* and *Lecanora conizaeoides*

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Abstract

Thallus pieces of the Mn-sensitive epiphytic lichen *Hypogymnia physodes* and of the Mn-resistant *Lecanora conizaeoides* were incubated in 5 mM $MnCl_2$ for 1 h. Element concentrations and thallus structure were subsequently studied with scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray microanalysis. Mn concentrations both in fungal and algal cell walls and cell lumina were much lower in *L. conizaeoides* than in *H. physodes*, because the former immobilized Mn in the thallus (e.g., in polyphosphate granules) and in apothecia. Within the apothecia, Mn was deposited with phosphate in the hypothecium and in an unknown form in the asci. Effective immobilization could cause the high Mn tolerance of *L. conizaeoides*. *H. physodes* also immobilized some Mn in extracellular particles in the upper cortex and in intracellular polyphosphate granules in the lower cortex. However, extra- and intracellular Mn concentrations in *H. physodes* increased much more during incubation with Mn compared to *L. conizaeoides*. The highest Mn concentrations were found in the upper and the lower cortex (i.e. in the cell walls and in the interhyphal polysaccharide matrix). The photobiont of *H. physodes* took up considerably less Mn than the mycobiont; this suggests that the latter is capable of protecting the photobiont to a certain extent from Mn invasion. Mn uptake released much Ca and Mg from *H. physodes*, especially from cortical cell walls and polysaccharide matrices. In the medulla, Mn was

incorporated in Ca oxalate crystals especially on the surface of young growing hyphae. On a long-term basis, this is suspected to affect the integrity of the crystals, which fulfill important structural and physiological functions. Mn exposure decreased the Fe/Mn ratio more in *H. physodes* than in *L. conizaeoides*. As Fe is known to alleviate Mn toxicity in *H. physodes*, this could be a mechanism causing the higher Mn sensitivity of this species. Si/Mn ratios decreased in all thallus layers of *H. physodes*, but not of *L. conizaeoides*; previous studies with soredia of *H. physodes* suggested possible alleviating effects of Si on Mn toxicity in lichens. Structural changes were observed in neither the mycobiont nor the photobiont of either lichen species.

1. Introduction

The foliose epiphytic lichen *Hypogymnia physodes* is sensitive to excess concentrations of Mn (HAUCK 2003). Negative correlations were found between the cover of *H. physodes* and the Mn concentrations in bark or stemflow in *Picea abies* forests (HAUCK et al. 2001b, 2002a). Mn concentrations in bark depend on the Mn content of the soil (HAUCK et al. 2002a), as Mn reaches the bark after root uptake, xylem transport and radial translocation (LÖVESTAM et al. 1990, SLOOF & WOLTERBEEK 1993). Leaching of Mn from foliage and bark primarily determines Mn concentrations in stemflow (LEVIA & HERWITZ 2000). Excess Mn caused significant chlorophyll degradation in soredia of *H. physodes* cultivated on agar plates as well as in thalli incubated with Mn salt solutions on a short-term basis (HAUCK et al. 2002d, 2003). Increasing Mn concentrations of the substrate increasingly inhibited the growth of soredia (HAUCK et al. 2002d). Ca, Mg, and Fe alleviated for Mn-induced growth inhibition of soredia and chlorophyll degradation (HAUCK et al. 2002d, 2003). Furthermore, Ca and Mg reduced the extracellular adsorption and the intracellular uptake of Mn in *H. physodes* (HAUCK et al. 2002c). Negative correlations of the abundance of *H. physodes* and other lichen species with the ratios of Mn to Ca, Mg or Fe in bark or in stemflow found in coniferous forests of Europe and North America support the significance of interactions between these elements in Mn toxicity (HAUCK et al. 2002a, HAUCK & SPRIBILLE 2002, SCHMULL & HAUCK 2003a).

The abundance of another frequent epiphyte on *Picea abies* in Europe, *Lecanora conizaeoides*, is not correlated with Mn concentrations in bark or stemflow (HAUCK et al. 2001c, 2002a). This species is known for its generally high toxitolerance (WIRTH 1985, HAUCK et al. 2001a). Short-term incubation with Mn concentrations as high as 10 mM did not affect chlorophyll concentrations in *L. conizaeoides* (HAUCK et al. 2003). Moreover, *L. conizaeoides* was found to take up less Mn from solution than *H. physodes* (HAUCK et al. 2002c).

In soredia of *H. physodes*, HAUCK et al. (2002c) studied structural changes subsequent to Mn exposure by means of scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Soredia developed shortened and swollen fungal hyphae and smaller and partly collapsed algal cells. The physical contact between the mycobiont and the photobiont was considerably reduced. This is supposed to impair the metabolite transfer between mycobiont and photobiont as well as the water supply of the photobiont (HONEGGER 1984, HONEGGER et al. 1996). Large amounts of Mn were immobilized in intracellular polyphosphate granules and in extracellular phosphate crusts. The latter completely covered some algal cells, resulting in their death. X-ray microanalysis showed that Mn uptake caused various changes in intra- and extracellular element concentrations, e.g., the loss of Ca and Mg from the mycobiont (HAUCK et al. 2002c). Higher Si/Mn ratios in the cell walls of living versus dead algal cells suggested a possible alleviating effect of Si in Mn toxicity in *H. physodes*; such an effect is known from vascular plants (EL-JAOUAL & COX 1998).

Since the chlorophyll measurements in thalli of *H. physodes* by HAUCK et al. (2003) showed that Mn exerts a detrimental effect not only on early developmental stages of *H. physodes*, but also on adult thalli, SEM and TEM studies of thalli of *H. physodes* were conducted in order to test the hypothesis that excess Mn causes structural damage both in the mycobiont and in the photobiont. By means of X-ray microanalysis, the hypothesis was tested that Mn is taken up in all parts of the thallus of *H. physodes*. Based on the proven interactions of Mn with Ca, Mg, Fe and P and on the assumed interaction with Si, we tested the hypothesis that Mn alters the concentrations of these elements in parts of the thallus. The Mn-tolerant *L. conizaeoides* was included in the study in order to test the hypothesis that Mn causes neither structural damage nor significant alterations in the Ca, Mg, Fe, P, and Si concentrations within the thallus. The

absence of such changes in element concentrations would explain the high resistance of the species.

2. Materials and methods

2.1. Incubation procedure and electron microscopy

Thalli of *H. physodes* (L.) Nyl. and *L. conizaeoides* Nyl. ex Crombie were sampled in the Göttingen area, Germany (51°33' N, 9°57' E). *H. physodes* has a stratified, heteromerous thallus, whereas the thallus of *L. conizaeoides* is homoiomerous. According to ITS rDNA sequencing (Helms, Hauck and Friedl, unpubl.), the photobiont of both species is *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner. Thalli of *H. physodes* were briefly moistened with water, put on paper towels, cleaned from bark and cut into pieces of about 1 cm^2 (i.e. of about 20 mg d. wt.). Thalli of the crustose *L. conizaeoides* were scraped of tree boles with a razor blade; thallus fragments obtained by this method weighed about 10 mg d. wt. Thallus pieces of each species were mixed in order to avoid effects of thallus-dependent differences in element concentrations. Thalli were stored in plastic bags at dark at 5-10 °C for one day prior to use. The thallus pieces of both lichens were preincubated on moist filter paper in Petri dishes for one day at 80 % relative humidity, a day temperature (for 13 hours daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1}, and a night temperature of 10 °C in the growth chamber. Thallus pieces of each species were gently shaken for 1 h with 5 mM $MnCl_2$. This concentration is in the range of total concentrations found in *Picea abies* bark of the Harz Mountains, Germany (HAUCK 2000); SCHMULL & HAUCK (2003a, b) showed that Mn is readily available from spruce bark. Samples incubated with deionized water served as controls. Subsequent to incubation, the samples were filtered and the lichen material was stored on moist filter paper in Petri dishes for one day in the growth chamber under climatic conditions as described above. With this day in the growth chamber following the exposure to the $MnCl_2$ solution, medium-term reactions of the lichen thalli could be studied, as this is ecologically more relevant compared to short-term reactions that may be reversible within a few hours.

Twenty thallus pieces per species with a lenght of 2 - 3 mm at each side were rapidly frozen in a 2 : 1 mixture of propane and isopentane cooled with liquid nitrogen to –196 °C, freeze-dried at –45 °C for 3 days and stored at 20 °C in a desiccator over silica gel. For SEM, the freeze-dried material was mounted on specimen holders (Cu grids), gold-coated and examined with a SEM 515 (Philips, Eindhoven, The Netherlands) operating at 20 kV. For TEM, freeze-dried thallus pieces were infiltrated with ether in a vacuum-pressure chamber and embedded in styrene-methacrylate (FRITZ 1989). Blocks were cut with dry glass knives with a Reichert ultramicrotome in ca. 1.0 µM thick sections. The sections were mounted on adhesive-coated 100-mesh hexagonal grids (FRITZ 1991), coated with carbon and stored over silica gel. The sections were analyzed in a Philips EM 420 with the energy dispersive system EDAX DX-4 (EDAX Inc., Mahwah, New Jersey, U.S.A.). The accelerating voltage was 120 kV, the take-off angle 25° and the counting time 60 live seconds. Quantitative data (mmol dm^{-3} element content) were obtained as described by FRITZ & JENTSCHKE (1994), taking into account the calibration coefficients (Cliff-Lorimer factors) of the elements relative to K. In the heteromerous *H. physodes*, EDAX measurements were carried out in the upper and lower cortex as well as in the algal layer and the medulla. The homoiomerous thallus of *L. conizaeoides* was (notionally) divided into three evenly thick horizontal layers (i.e., upper and lower thallus, thallus centre) using the apothecia as a guide for orientation within the thallus.

2.2. Statistics

Arithmetic means ± standard error are given throughout the paper. Data were tested for normal distribution with the Shapiro-Wilk test and tested for significant differences with Duncan's multiple range test (for comparisons of more than two means). Statistical analyses were computed with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.).

3. Results

Mn exposure increased the Mn concentrations in all parts of the thallus of *H. physodes* (Table 6-1). Mn concentrations in the upper and the lower cortex were much higher compared to the algal layer and the medulla. In the upper cortex, most Mn was

deposited in small extracellular particles (Fig. 6-1C), which were poor in phosphate and sulphate, but in rich in chloride (Table 6-2). Furthermore, high amounts of Mn were located in the interhyphal polysaccharide matrix and in the fungal cell walls (Table 6-2). In the lower cortex of Mn-exposed samples, Mn concentrations were also higher in the cell walls (103 ± 11 mmol dm^{-3}) and in the matrix material (168 ± 16 mmol dm^{-3}) than in the cell interior (37.7 ± 21.7 mmol dm^{-3}). Mn-rich extracellular particles were not observed in the lower cortex, but moderate amounts of Mn were accumulated intracellularly in polyphosphate granules (Mn: 89.5 ± 19.4 mmol dm^{-3}, P: 592 ± 142 mmol dm^{-3}). The fungal cell walls of Mn-treated samples contained much less Mn in the medulla than in the cortex, whereas intracellular Mn concentrations were in the same range in hyphae of these layers (Table 6-3). While the polysaccharide matrix is restricted to the cortex, the medulla of *H. physodes* is characterized by numerous Ca oxalate crystals covering the surface of the hyphae (Fig. 6-1D). While old hyphae were completely surrounded by crystals (Fig. 6-1E), young parts of the hyphae were only patchily covered (Fig. 6-1F). Because of these crystals, very high (molar) Ca concentrations occurred in the medulla (Table 6-1). Mn was deposited in the oxalate crystals decreasing their Ca/Mn ratios (Table 6-4). Significantly more Mn was recovered in the crystals on young versus old hyphae. The number of crystals in Mn-treated samples was not different from control samples as visually assessed with SEM (Figs. 6-2C, D) and TEM. Algal cells took up less Mn intracellularly and adsorped less Mn at the cell walls compared to the fungal cells of any layer (Table 6-3). Mn deposits in polyphosphate granules or other particles were not observed in the photobiont.

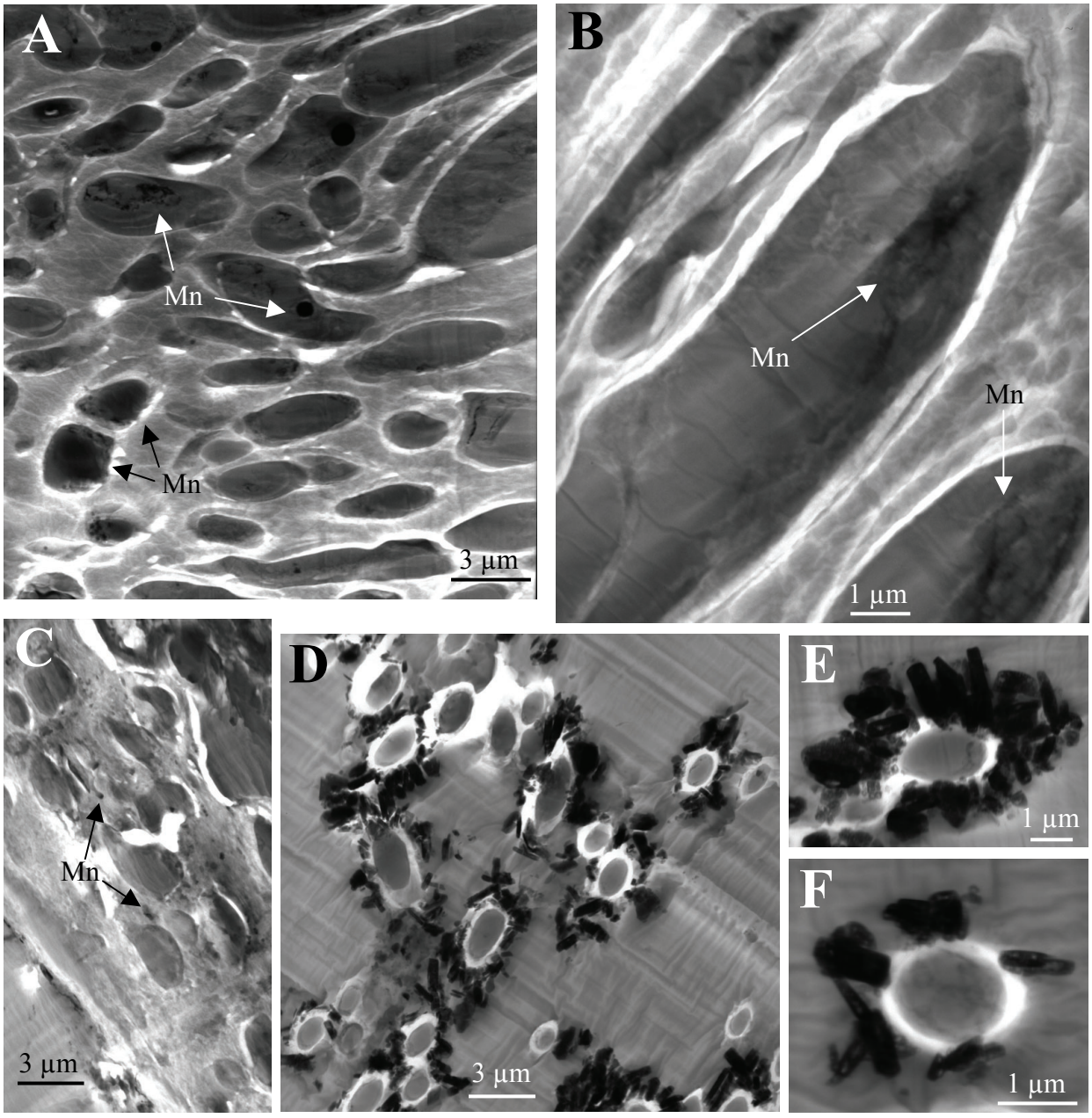

Fig. 6-1. TEM micrographs of semithin sections of *L. conizaeoides* (A, B) and *H. physodes* (C - F) subsequent to incubation with 5 mM $MnCl_2$ for 1 h. A. Hypothecium with electron-dense Mn deposits (Mn). B. Asci with Mn deposits. C. Extracellular Mn deposits in the upper cortex. D - F. Ca oxalate crystals around hyphae in the medulla. E. Old hypha with dense cover of Ca oxalate crystals. F. Young hypha with scattered Ca oxalate crystals on the surface.

Table 6-1. Element concentrations (in mmol dm^{-3}) in *Hypogymnia physodes* subsequent to 1 h exposure to 5 mM $MnCl_2$[a]

	Upper cortex		Algal layer		Medulla		Lower cortex	
Control:								
Mn	1.59 ± 0.25	a	0.26 ± 0.14	b	0.78 ± 0.29	b	2.67 ± 0.18	a
K	165 ± 9	a	72.8 ± 6.5	b	44.1 ± 4.2	b	231 ± 17	a
Ca	149 ± 10	a	11.4 ± 4.16	a	2530 ± 360	b	157 ± 10	a
Mg	130 ± 9	a	23.6 ± 3.0	b	14.1 ± 2.5	b	110 ± 8	a
Fe	1.29 ± 0.20	a	1.36 ± 0.21	a	1.42 ± 0.28	a	6.40 ± 0.90	b
Si	16.1 ± 8.9	ab	10.2 ± 0.2	a	22.2 ± 2.4	b	23.2 ± 4.0	b
P	25.1 ± 3.1	a	27.1 ± 1.8	a	18.5 ± 2.8	a	72.2 ± 7.6	b
S	15.7 ± 1.6	a	31.2 ± 3.1	b	8.47 ± 1.06	c	29.7 ± 1.6	b
Cl	30.4 ± 4.3	a	9.25 ± 1.09	b	10.4 ± 3.5	b	58.1 ± 5.6	c
Ca/Mn	85.9 ± 7.4	a	32.3 ± 11.6	a	1170 ± 130	b	59.5 ± 2.7	a
Mg/Mn	73.5 ± 8.0	a	29.4 ± 3.2	bc	7.70 ± 1.10	b	42.6 ± 4.0	ac
Fe/Mn	0.72 ± 0.02	a	2.51 ± 0.81	b	1.16 ± 0.22	a	2.45 ± 0.39	b
Si/Mn	9.47 ± 1.14	a	11.0 ± 1.5	a	11.6 ± 1.7	a	9.45 ± 2.27	a
Mn 5 mM:								
Mn	141 ± 10	a*	13.1 ± 8.5	b	6.82 ± 1.02	b*	103 ± 11	c*
K	134 ± 15	a	93.7 ± 3.4	ac*	22.6 ± 3.05	b*	80.9 ± 7.7	c*
Ca	61.8 ± 4.4	a*	4.69 ± 2.91	a	1620 ± 390	b	62.4 ± 6.3	a*
Mg	42.8 ± 4.7	a*	34.3 ± 2.6	a*	5.60 ± 1.38	b*	14.4 ± 2.0	b*
Fe	5.64 ± 1.93	a*	2.63 ± 0.43	a*	1.48 ± 0.28	a	27.4 ± 6.6	b*
Si	15.0 ± 1.8	ab	8.63 ± 0.49	a	13.0 ± 1.9	ab*	24.9 ± 8.1	b
P	25.5 ± 3.0	a	34.1 ± 2.6	a*	9.43 ± 2.02	b*	8.61 ± 2.17	b*
S	40.1 ± 4.6	a*	55.4 ± 4.1	b*	13.1 ± 1.6	c*	30.9 ± 6.1	a
Cl	55.2 ± 4.7	a*	11.2 ± 2.5	b	10.1 ± 1.7	b	57.7 ± 5.4	a
Ca/Mn	0.44 ± 0.01	a*	0.43 ± 0.11	a	356 ± 128	b*	0.62 ± 0.02	a*
Mg/Mn	0.31 ± 0.04	a*	6.64 ± 1.45	b*	1.26 ± 0.35	a*	0.15 ± 0.02	a*
Fe/Mn	0.05 ± 0.10	a*	0.58 ± 0.16	b*	0.28 ± 0.07	c*	0.25 ± 0.05	c*
Si/Mn	0.11 ± 0.01	a*	1.73 ± 0.49	bc*	3.06 ± 0.93	b*	0.29 ± 0.09	ac*

[a] Arithmetic mean ± standard error. Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 45$ [control], 44 [5 mM $MnCl_2$]). Asterisks indicate significant difference of the respective element concentration or element ratio at 5 mM $MnCl_2$ to the control (Duncan's multiple range test, $P \leq 0.05$, $df = 37$ [upper cortex], 14 [algal layer], 22 [medulla], 16 [lower cortex]). Number of samples, control/5 mM $MnCl_2$: upper cortex, 19/20; algal layer, 10/6; medulla, 5/12; lower cortex, 8/10.

Mn uptake resulted in a significant decrease of Ca concentrations in the upper and the lower cortex as well as of Mg concentrations in all thallus layers of *H. physodes* (Table 6-1). The decreases in the cortices were confined to the cell walls and the polysaccharide matrix (Table 6-2). Mn reduced the Mg concentrations in the medulla to a substantially lower degree than in the cortex (Table 6-3). The Ca and Mg concentrations in the photobiont were not affected by Mn (Table 6-3). The ratios of Ca and Mg to Mn decreased everywhere in the mycobiont and in the algal cell walls, but not in the algal cell lumina (Table 6-3). Fe concentrations increased in the cortices and in the algal layer, but remained constant in the medulla (Table 6-1). The increases in the former layers were due to increased extracellular Fe concentrations in the cortical hyphae and in the photobiont in particular (Tables 6-2, 6-3). The Fe/Mn ratios, however, decreased both extra- and intra-cellularly in the fungus and in the alga (Tables 6-1 – 6-3). The Si/Mn ratios also decreased in most parts of the thallus, whereas the Si concentrations in Mn-treated samples were mostly not significantly different from the control. Mn reduced the concentration of P in the medulla, viz. primarily in the cell walls (Tables 6-1, 6-3), whereas the P concentration in the algal layer increased slightly. The Mn treatment caused no structural changes in *H. physodes*; the photobiont cells as well as the fungal hyphae remained intact without showing any changes in shape or size (Figs. 6-2A, B, E, I). The physical contact between mycobiont and photobiont was not affected.

Table 6-2. Element concentrations (in mmol dm^{-3}) in the fungal cell walls and cell lumina as well as in the interhyphal matrix and in extracellular Mn deposits of the upper cortex in *Hypogymnia physodes* subsequent to 1 h exposure to 5 mM $MnCl_2$[a]

	Cell walls		Cell lumina		Matrix		Mn deposits	
Control:								
Mn	2.09 ± 0.38	a	0.46 ± 0.31	b	2.59 ± 0.37	a		
K	216 ± 13	a	61.1 ± 16.6	b	169 ± 45	a		
Ca	189 ± 10	a	15.9 ± 5.4	b	217 ± 43	a		
Mg	215 ± 14	a	46.6 ± 12.5	b	151 ± 27	c		
Fe	1.63 ± 0.42	a	2.40 ± 0.74	a	1.56 ± 0.23	a		
Si	10.3 ± 1.2	a	13.2 ± 2.4	ab	17.6 ± 1.5	b		
P	8.43 ± 2.67	a	46.7 ± 17.5	b	9.47 ± 6.58	a		
S	5.92 ± 0.77	a	34.5 ± 7.5	b	6.66 ± 2.48	a		
Cl	23.7 ± 4.5	a	20.6 ± 13.6	a	27.8 ± 24.0	a		
Ca/Mn	102 ± 15	a	6.50 ± 0.05	b	80.9 ± 6.4	a		
Mg/Mn	115 ± 16	a	21.8 ± 9.9	b	57.1 ± 3.9	ab		
Fe/Mn	0.83 ± 0.20	a	2.02 ± 0.18	b	0.66 ± 0.14	a		
Si/Mn	5.85 ± 1.19	a	4.17 ± 1.85	a	7.43 ± 1.04	a		
Mn 5 mM:								
Mn	220 ± 30	a*	14.7 ± 5.0	b*	236 ± 34	a*	367 ± 17	c
K	188 ± 24	a	52.3 ± 12.8	b	233 ± 54	ac	361 ± 24	c
Ca	78.9 ± 8.5	a*	6.52 ± 2.22	b	112 ± 17	ac*	130 ± 6	c
Mg	61.0 ± 11.9	ab*	97.2 ± 34.8	a	44.2 ± 6.1	b*	154 ± 21	c
Fe	2.04 ± 0.36	a	2.08 ± 0.30	a	6.04 ± 2.81	a	2.14 ± 1.35	a
Si	26.5 ± 1.0	a*	18.7 ± 1.4	a	20.5 ± 2.0	a	39.8 ± 7.7	b
P	14.1 ± 7.4	a	70.9 ± 12.8	b	6.20 ± 1.79	a	11.9 ± 3.1	a
S	21.9 ± 0.7	a*	41.4 ± 5.7	b	13.7 ± 2.0	a*	35.7 ± 7.6	b
Cl	28.5 ± 3.8	a	7.37 ± 0.69	a	34.9 ± 6.0	a	481 ± 128	b
Ca/Mn	0.35 ± 0.01	a*	0.43 ± 0.07	ab*	0.47 ± 0.00	b*	0.36 ± 0.01	a
Mg/Mn	0.30 ± 0.02	a*	7.84 ± 1.83	b*	0.20 ± 0.02	a*	0.42 ± 0.05	a
Fe/Mn	0.02 ± 0.02	a*	0.20 ± 0.04	b*	0.03 ± 0.01	a*	0.01 ± 0.00	a
Si/Mn	0.17 ± 0.03	a*	1.86 ± 0.28	b*	0.13 ± 0.03	a*	0.20 ± 0.02	a

[a] Arithmetic mean ± standard error. Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 23$[control], 38 [5 mM $MnCl_2$]). Asterisks indicate significant difference of the respective element concentration or element ratio at 5 mM $MnCl_2$ to the control (Duncan's multiple range test, $P \leq 0.05$, $df = 24$ [cell walls], 14 [cell lumina], 19 [matrix]). Number of samples, control/5 mM $MnCl_2$: cell walls, 11/15; cell lumina, 8/8; matrix, 7/14; Mn deposits, 0/5.

Table 6-3. Element concentrations (in mmol dm^{-3}) in the cell walls and cell lumina of the photobiont (*Trebouxia jamesii*) as well as in the cell walls and cell lumina of medulla hyphae in *Hypogymnia physodes* subsequent to 1 h exposure to 5 mM $MnCl_2$[a]

	Trebouxia jamesii				Medulla hyphae			
	Cell walls		Cell lumina		Cell walls		Cell lumina	
Control:								
Mn	0.59 ± 0.17	a	0.55 ± 0.15	a	1.26 ± 0.36	a	0.83 ± 0.29	a
K	103 ± 13	a	61.0 ± 4.2	a	200 ± 50	b	93.9 ± 21.8	a
Ca	3.17 ± 0.80	a	1.14 ± 0.33	a	33.6 ± 5.3	b	48.3 ± 13.0	c
Mg	23.8 ± 5.1	a	24.8 ± 2.7	a	31.6 ± 10.2	a	22.2 ± 8.0	a
Fe	0.94 ± 0.13	a	2.44 ± 0.20	b	1.92 ± 0.31	bc	1.42 ± 0.34	ac
Si	24.5 ± 5.3	a	12.3 ± 2.6	ab	16.6 ± 5.6	ab	6.89 ± 2.02	a
P	34.1 ± 8.6	a	37.3 ± 1.2	a	148 ± 53	b	129 ± 63	b
S	28.7 ± 3.8	ac	70.5 ± 5.7	b	41.9 ± 7.3	a	20.6 ± 2.9	c
Cl	19.7 ± 4.0	a	6.23 ± 1.74	ab	13.7 ± 10.5	ab	1.31 ± 0.68	b
Ca/Mn	1.99 ± 0.72	a	1.45 ± 0.54	a	22.1 ± 4.0	b	27.6 ± 3.5	b
Mg/Mn	30.2 ± 6.0	a	33.6 ± 7.9	a	23.0 ± 8.6	a	24.2 ± 11.6	a
Fe/Mn	1.57 ± 0.38	a	3.15 ± 0.53	b	1.61 ± 0.22	a	1.44 ± 0.20	a
Si/Mn	22.8 ± 5.3	a	17.5 ± 4.8	a	13.6 ± 7.1	a	6.89 ± 2.44	a
Mn 5 mM:								
Mn	6.26 ± 0.68	a*	1.37 ± 0.34	a	29.1 ± 5.3	b*	16.4 ± 4.5	c*
K	116 ± 17	ab	65.3 ± 3.7	ac	149 ± 33	b	28.6 ± 7.9	c*
Ca	2.90 ± 0.34	a	1.47 ± 0.25	a	91.0 ± 39.8	b	31.5 ± 4.1	a
Mg	19.1 ± 3.6	a	30.1 ± 7.7	a	16.7 ± 2.68	a	12.2 ± 2.9	a
Fe	1.80 ± 0.19	a*	2.71 ± 0.32	b	1.05 ± 0.36	a	1.66 ± 0.14	a
Si	19.8 ± 1.8	a	11.8 ± 1.1	b	17.5 ± 4.5	ab	9.84 ± 1.79	b
P	28.9 ± 5.7	a	38.9 ± 1.1	a	56.3 ± 13.4	a	94.5 ± 26.9	b
S	38.5 ± 5.0	a	74.9 ± 13.2	b	36.4 ± 3.6	a	37.7 ± 7.7	a
Cl	27.3 ± 2.8	a	13.3 ± 1.9	a*	27.2 ± 3.6	a	25.0 ± 13.2	a
Ca/Mn	0.52 ± 0.07	a*	1.02 ± 0.26	a	3.41 ± 1.44	b*	2.82 ± 0.61	b*
Mg/Mn	2.96 ± 0.51	a*	19.4 ± 6.7	b	0.72 ± 0.16	a*	0.95 ± 0.19	a*
Fe/Mn	1.10 ± 0.05	a*	1.86 ± 0.27	b*	0.08 ± 0.05	a*	0.17 ± 0.05	a*
Si/Mn	4.14 ± 0.57	a*	8.39 ± 4.00	b*	0.69 ± 0.14	c	1.27 ± 0.56	c*

[a] Arithmetic mean ± standard error. Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 34$ [control], 54 [5 mM $MnCl_2$]). Asterisks indicate significant difference of the respective element concentration or element ratio at 5 mM $MnCl_2$ to the control (Duncan's multiple range test, $P \leq 0.05$, $df = 41$ [algal cell walls], 23 [algal cell lumina], 12 [fungal cell walls], 12 [fungal cell lumina]). Number of samples, control/5 mM $MnCl_2$: algal cell walls, 15/28; algal cell lumina, 11/14; fungal cell walls, 6/8; fungal cell lumina, 6/8.

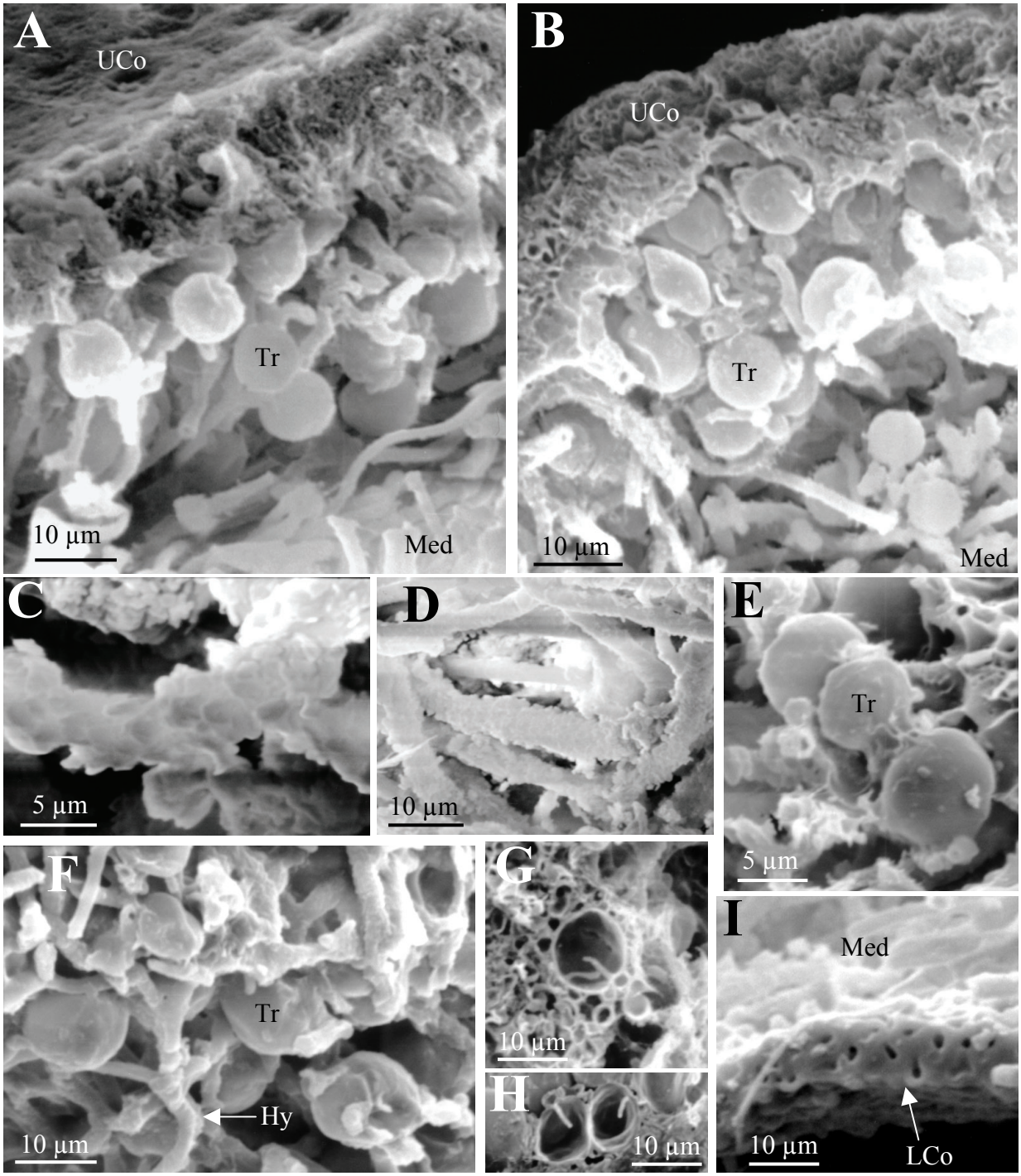

Fig. 6-2. SEM micrographs of *H. physodes* (A – E, I) and *L. conizaeoides* (F - H) subsequent to incubation with 5 mM $MnCl_2$ (B, D – F, I) or deionized water (A, C, G) for 1 h. A. Upper cortex (UCo), photobiont cells (*Trebouxia jamesii*; Tr) in the algal layer and hyphae of the medulla (Med). B. Treatment with $MnCl_2$ causes no structural changes compared to control. C, D. Hyphae in the medulla densely covered with Ca oxalate crystals. E. Intact photobiont cells. F. Intact mycobiont hyphae (Hy) and *Trebouxia jamesii* cells. G, H. Intracellular haustoria in cross-section. I. Lower cortex (LCo) and medulla.

L. conizaeoides contained much less Mn subsequent to the incubation with Mn than *H. physodes* (Table 6-5). Mn concentrations in *L. conizaeoides* were in the same range as in the medulla of *H. physodes*, but were half as much as in the algal layer and 15 - 25 times lower than in the cortices of *H. physodes*. Mn was evenly distributed within the vegetative thallus parts of Mn-exposed *L. conizaeoides* samples. Fungal cell walls (7.30 ± 1.05 mmol dm^{-3}) and cell lumina (1.54 ± 0.52 mmol dm^{-3}) contained much less Mn compared to hyphae of *H. physodes*. In the algal cells of Mn-exposed *L. conizaeoides*, Mn concentrations amounted to less than 50 % of the corresponding concentrations in *H. physodes* in the cell walls (2.73 ± 0.78 mmol dm^{-3}) and to less than 30 % in the cell lumina (0.37 ± 0.16 mmol dm^{-3}). Mn uptake decreased the ratios of Ca, Mg, and Fe to Mn in all thallus parts of *L. conizaeoides*, whereas the Si/Mn ratio remained constant (Table 6-5). The concentrations of Fe and Si significantly increased in the entire thallus, whereas the concentrations of Mg and P increased only in the upper thallus; the concentration of Ca decreased in the lower thallus.

Table 6-4. Element concentrations (in mmol dm^{-3}) in oxalate crystals on mycobiont hyphae in the medulla of *Hypogymnia physodes* subsequent to 1 h exposure to 5 mM $MnCl_2$[a]

	Control	Mn 5 mM	
	mature hyphae	mature hyphae	young hyphae
Mn	0.56 ± 0.25 a	9.70 ± 6.12 a	23.4 ± 2.0 b
K	11.4 ± 2.5 a	43.8 ± 30.6 ab	128 ± 27 b
Na	1.52 ± 1.00 a	10.9 ± 4.5 ab	18.3 ± 6.0 b
Ca	3080 ± 710 a	2670 ± 390 a	1730 ± 430 a
Mg	5.81 ± 1.49 a	12.7 ± 2.2 ab	17.3 ± 4.5 b
Ca/Mn	2310 ± 580 a	793 ± 269 b	76.5 ± 17.7 b

[a] Arithmetic mean ± standard error. Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 17$). Number of samples: control, $n = 8$; Mn 5 mM, mature hyphae, $n = 6$; young hyphae, $n = 6$.

Table 6-5. Element concentrations (in mmol dm^{-3}) in the thallus of *Lecanora conizaeoides* subsequent to 1 h exposure to 5 mM $MnCl_2$[a]

	Upper thallus		Centre		Lower thallus	
Control:						
Mn	0.22 ± 0.10	a	0.60 ± 0.18	b	0.22 ± 0.14	a
K	71.5 ± 7.8	a	62.9 ± 5.4	a	49.7 ± 8.2	a
Ca	6.55 ± 3.81	a	1.72 ± 0.32	a	2.83 ± 0.64	a
Mg	7.81 ± 0.95	a	8.13 ± 0.90	a	5.77 ± 1.85	a
Fe	2.58 ± 0.42	a	5.78 ± 0.89	b	4.42 ± 1.07	ab
Si	2.99 ± 2.99	a	9.59 ± 2.04	a	3.98 ± 0.82	a
P	17.2 ± 1.09	a	16.4 ± 3.0	b	13.8 ± 3.0	a
S	31.0 ± 1.9	a	28.9 ± 3.2	a	22.3 ± 3.4	a
Cl	22.1 ± 10.1	a	13.9 ± 1.8	a	29.5 ± 15.1	a
Ca/Mn	3.44 ± 0.86	a	2.26 ± 0.66	a	3.07 ± 2.00	a
Mg/Mn	12.7 ± 1.7	a	10.1 ± 1.0	a	15.4 ± 1.24	a
Fe/Mn	3.46 ± 0.65	a	7.03 ± 2.26	a	6.21 ± 0.76	a
Si/Mn	9.12 ± 1.85	a	12.2 ± 2.3	a	3.47 ± 3.47	a
Mn 5 mM:						
Mn	6.94 ± 1.25	a*	6.65 ± 0.86	a*	5.36 ± 0.73	a*
K	36.8 ± 5.5	a*	35.2 ± 5.5	a*	54.3 ± 11.8	a
Ca	1.97 ± 0.41	a	1.18 ± 0.36	a	1.04 ± 0.39	a*
Mg	12.9 ± 1.3	a*	5.99 ± 1.47	b	14.1 ± 2.6	a
Fe	11.0 ± 3.2	a*	10.8 ± 2.0	a*	4.81 ± 0.62	a
Si	39.5 ± 9.0	a*	40.0 ± 4.7	a*	34.3 ± 6.0	a*
P	26.4 ± 3.3	a*	18.5 ± 2.5	a	19.0 ± 2.2	a
S	34.2 ± 3.4	a	31.3 ± 2.3	a	24.5 ± 3.6	a
Cl	8.52 ± 1.61	a	10.1 ± 1.0	a	8.67 ± 1.78	a
Ca/Mn	0.38 ± 0.10	a*	0.28 ± 0.10	a*	0.21 ± 0.07	a*
Mg/Mn	2.91 ± 0.53	a*	1.74 ± 0.59	a*	2.88 ± 0.47	a*
Fe/Mn	1.24 ± 0.21	a*	1.59 ±0.20	a*	1.02 ± 0.17	a*
Si/Mn	5.26 ± 0.73	a	8.16 ± 2.67	a	6.50 ± 1.04	a

[a] Arithmetic mean ± standard error. Within a row, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$; control: $df = 28$, 5 mM $MnCl_2$: $df = 39$). Asterisks indicate significant difference of the respective element concentration or element ratio at 5 mM $MnCl_2$ to the control (Duncan's multiple range test, $P \leq 0.05$, $df = 26$ [upper thallus], 24 [centre], 17 [lower thallus]). Number of samples, control/5 mM $MnCl_2$: upper thallus, 13/15; centre, 12/14; lower thallus, 6/13.

Considerable amounts of Mn were accumulated in the apothecia of Mn-treated *L. conizaeoides* samples. Electrone-dense areas were found in the hypothecium (Fig. 6-1A) with a mean Mn concentration of 401 ± 109 mmol dm^{-3}; these areas further contained significant amounts of K (511 ± 64 mmol dm^{-3}) and Mg (239 ± 17 mmol dm^{-}

^{3}). In these deposits, most Mn was probably complexed with phosphate, as P was measured in a concentration of 1380 ± 100 mmol dm^{-3}. Furthermore, Mn deposits were found in the asci (222 ± 42 mmol dm^{-3}; Fig. 6-1B). As in the hypothecium, the high Mn concentrations were correlated with high concentrations of K (434 ± 51 mmol dm^{-3}) and Mg (129 ± 20 mmol dm^{-3}). While the P concentrations in the asci were comparably low (178 ± 22 mmol dm^{-3}), the S concentration amounted to 1020 ± 220 mmol dm^{-3}. The Mn concentrations in the excipulum (6.44 ± 1.45 mmol dm^{-3}) were as high as in the vegetative parts of the thallus. In the vegetative thallus, moderate amounts of Mn were accumulated intracellularly in the mycobiont in polyphosphate granules; an Mn concentration of 36.1 ± 10.8 mmol dm^{-3} was measured in a granule containing P in a concentration of 997 ± 311 mmol dm^{-3}). Other intracellular particles in the mycobiont contained much more Mn (536 ± 30 mmol dm^{-3}). However, the chemical composition of these particles is unknown, as the contained only 72.6 ± 22.5 mmol dm^{-3} of P, 290 ± 13 mmol dm^{-3} of S, and 11.6 ± 2.4 mmol dm^{-3} of Cl, but high amounts of Ca (2840 ± 124 mmol dm^{-3}) and K (1140 ± 53 mmol dm^{-3}). Structural damage due to the Mn treatment could not be observed with SEM (Fig. 6-2F). Intracellular haustoria were observed as frequent as in the control (Figs. 6-2 G, H).

4. Discussion

The present study confirms findings of HAUCK et al. (2002c) obtained by means of sequential elution with water and EDTA and subsequent acid digestion that *L. conizaeoides* takes up less Mn from solution than *H. physodes*. Furthermore, *L. conizaeoides* is apparently capable of keeping physiologically relevant Mn concentrations on a low level by the immobilization of Mn in the mycobiont. Remarkably, Mn deposits were found especially frequently in the apothecia, viz. in the hypothecium and in the asci. This result parallels findings in *Lecidella bullata* (Fam. *Lecanoraceae*), where Cu was accumulated in the hypothecium and in a pruina on the disc surface (PURVIS et al. 1990). Cu accumulation also occurred in apothecia of *Lecanora polytropa* (ALSTRUP & HANSEN 1977). X-ray microanalysis suggests that Mn deposits of different chemical constitution occur in *L. conizaeoides*. In vegetative parts of the thallus, Mn was found in polyphosphate granules and in an unknown type of particles. Mn immobilization in polyphosphate granules has already been observed in soredia of *H.*

physodes (HAUCK et al. 2002d). In the hypothecium, Mn was also immobilized with phosphate. Nonetheless, it should be tested whether complexation with the depsidone fumarprotocetraric acid is involved in the immobilization of Mn in *L. conizaeoides*. Fumarprotocetraric acid is the major lichen substance in *L. conizaeoides* (KÜMMERLING 1991). The depsidone psoromic acid was found to be the anionic compound complexing Cu in the apothecia of *Lecidella bullata* (PURVIS et al. 1990). Complexes of Cu with psoromic acid and a further depsidone, norstictic acid, were found in several lichen species (PURVIS et al. 1987, 1990). The three depsidones in question have an *ortho*-phenolic hydroxyl group and an adjacent aldehyde group in common (HUNECK & YOSHIMURA 1996). These functional groups, which are the assumed binding sites in the complexes of Cu with psoromic and norstictic acid (PURVIS et al. 1990), perhaps also bind to Mn. Since complexes of lichen substances with Mn, or of norstictic acid with metal ions, have not been found so far, further study is needed.

While *L. conizaeoides* maintained low Mn concentrations in the cell lumina as well as in the cell walls both of the mycobiont and the photobiont, Mn concentrations in *H. physodes* were strongly dependent on the location within the thallus. The lowest Mn concentrations occurred in the algal cell lumina, followed by the algal cell walls. This suggests that the mycobiont in *H. physodes* may have the ability to protect the photobiont to a certain extent from the invasion of Mn. The photobiont is usually thought to be the most sensitive part of the lichen symbiosis, because the mycobiont enhances the permeability of the algal or cyanobacterial membranes for assimilate transfer (AHMADJIAN 1993). In contrast to the mycobiont, the intracellular Ca/Mn and Mg/Mn ratios in the photobiont were not affected by Mn. In the fungal cells of *H. physodes*, the Mn concentrations increased significantly. Most Mn was bound to the cortical surfaces of *H. physodes*. Fungal cell walls provide many anionic exchange sites such as carboxyl groups (RICHARDSON 1995). Extracellular polysaccharides formed by lichens also harbour carboxyl groups and other anionic binding sites (MODENESI & VANZO 1986, MODENESI et al. 1986). Under field conditions, most extracellular exchange sites in lichens are usually charged with Ca and Mg (BROWN & BROWN 1991). This explains why primarily Ca and Mg were released both from the cell walls and from the polysaccharide matrix during Mn adsorption (HAUCK et al. 2002c). In contrast to *L. conizaeoides*, the Si/Mn ratios were significantly reduced in the thallus of *H. physodes*, which may result in higher Mn sensitivity of the latter (HAUCK et al. 2002d). The en-

richment of Si in *L. conizaeoides* during the incubation may result from very small residual substrate pieces, which could not be removed completely from the thallus before the experiment. However, another possible explanation is that the samples were somewhat heterogenous, though they were all collected from the same site and mixed before the incubation procedure. Higher Si and S concentrations in some thallus parts of the Mn-treated *H. physodes* samples than in the control support the latter explanation.

The accumulation of Mn in the Ca oxalate crystals in the medulla of *H. physodes* may be detrimental, because impurities could alter the crystal structure and integrity (MODENESI et al. 1997). Since the Mn concentration was highest in oxalate crystals on the surface of young growing hyphae, Mn can presumably influence the formation of new crystals (CODY & HORNER 1984). The Ca oxalate crystals in the medulla fulfill various structural and physiological functions. The crystals in *H. physodes*, consisting primarily of Ca oxalate monohydrate (whewellite), provide mechanical stability to the medulla with its large cavities, which are thought to be important for the diffusion of gases (MODENESI et al. 1997). Furthermore, the crystals in the medulla are supposed to reflect light that already passed the algal layer and, thus, to increase the photosynthetic quantum yield (MODENESI et al. 2000, CLARK et al. 2001).

The short-term exposure to Mn did not result in structural damage in neither *H. physodes* nor *L. conizaeoides*. Since HAUCK et al. (2002d) found numerous damage symptoms in soredia of *H. physodes* cultivated on agar plates with 7 mM $MnCl_2$ for 8 days, an experiment with thalli repeatedly sprayed with low-concentrated Mn solutions on a long-term basis should be carried out in order to test whether adult thalli of epiphytic lichens suffer structural damage when they are exposed to Mn for extended periods.

In conclusion, the study suggests that *L. conizaeoides* is capable of maintaining much lower Mn levels in the cell lumina and in the cell walls compared to *H. physodes*. The high amounts of released Ca and Mg in *H. physodes* could affect, e.g., the stability of cell walls and membranes, but could also disturb various intracellular processes (HAUCK et al. 2002c). Such considerations are consistent with the observed decreasing abundance of *H. physodes* found with increasing Mn/Ca ratio of stemflow in the field (HAUCK et al. 2002a). The Fe/Mn ratios were much higher in *L. conizaeoides* versus *H.*

physodes. This can be a major cause for the different Mn sensitivity of the two species, because HAUCK et al. (2003) showed that Fe has a strongly alleviating on Mn toxicity in *H. physodes*. Though Mn was shown to be immobilized with phosphate also in the present study, P does not appear to be a limiting factor for the abundance of either lichen species; HAUCK et al. (2003) showed that incubation of *H. physodes* with 10 mM $MnCl_2$ for 1 h did not result in ATP deficiency. Translocation of P from the medulla to the algal layer in *H. physodes* (Table 6-1) may indicate that P was used there for the immobilization of Mn in order to protect the photobiont from toxic Mn concentrations. Higher Si/Mn ratios in *L. conizaeoides* than in *H. physodes* suggest that Si is involved in Mn tolerance in lichens, as already suggested by HAUCK et al. (2002d) because of higher Si/Mn ratios in living versus collapsed photobiont cells in Mn-treated *H. physodes* soredia.

Chapter 7

Manganese toxicity in epiphytic lichens: chlorophyll degradation and interaction with iron and phosphorus

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Abstract

Concentrations of chlorophylls *a* and *b* decreased with increasing $MnCl_2$ supply in the epiphytic lichen *Hypogymnia physodes*, but not in *Lecanora conizaeoides*. The reduction of chlorophyll concentrations in *H. physodes* was as strong (chlorophyll *a*) or even stronger (chlorophyll *b*) as in samples treated with $CuCl_2$. $FeCl_3$ compensated for $MnCl_2$-induced chlorophyll degradation in *H. physodes*. Furthermore, $MnCl_2$-induced growth inhibition of soredia cultivated on agar plates was alleviated by $FeCl_3$. These results suggest that Mn causes intracellular Fe deficiency in *H. physodes*. A soredia growth test with $MnCl_2$ and KCl in combination proved that mitigating effects of $FeCl_3$ were not just caused by reduced chemical activity of Mn^{2+} due to the addition of another salt. Furthermore, the test showed that Cl^- did not inhibit soredia growth. High $FeCl_3$ concentrations applied alone or in combination with $MnCl_2$ were even more detrimental to *H. physodes* than $MnCl_2$. $MnCl_2$ did not affect the concentrations of ATP, ADP and AMP in *H. physodes*. This suggests that Mn uptake does not induce intracellular P deficiency in *H. physodes* despite that Mn is known to be immobilized with P in *H. physodes* in intracellular polyphosphate granules and in extracellular encrustations.

1. Introduction

Excess Mn reduces the concentrations of the chlorophylls *a* and *b* in soredia of the foliose epiphytic lichen *Hypogymnia physodes* cultivated on agar plates. The chlorophyll content is correlated with the ability of the soredia to grow (HAUCK et al. 2002d). These findings agree with field data showing decreasing cover of *H. physodes* with increasing Mn concentrations in bark and stemflow of *Picea abies* (HAUCK et al. 2001b, 2002a). In the present study, we examined whether Mn-induced chlorophyll degradation is limited to the early development of *H. physodes*. Therefore, short-term incubation experiments with thallus pieces were carried out in order to test the hypothesis that Mn also reduces chlorophyll concentrations in completely developed thalli of *H. physodes*. In contrast to *H. physodes*, the cover of the crustose lichen *Lecanora conizaeoides* is not correlated with the concentrations of Mn in bark or stemflow (HAUCK et al. 2001b, 2002a). We included this lichen species in the study in order to test the hypothesis that chlorophyll concentrations in *L. conizaeoides* are less affected by Mn than in *H. physodes*. Since Ca and Mg alleviate Mn-induced growth inhibition in soredia of *H. physodes* and, further, reduce the extracellular adsorption and the intracellular uptake of Mn in *H. physodes* (HAUCK et al. 2002c, d), we tested the hypothesis that Ca and Mg compensate for Mn-induced reductions of chlorophyll concentrations in *H. physodes*. Mn-induced Fe deficiency is known to be a possible cause for reduced chlorophyll concentrations in vascular plants, as Fe is essential for chlorophyll biosynthesis (HORST 1988, EL-JAOUAL & COX 1998). Therefore, we tested the hypothesis that Fe compensates for Mn-induced reduction of the chlorophyll concentrations in *H. physodes*. Because of the known correlation between soredia growth and chlorophyll content (HAUCK et al. 2002d), we also tested the hypothesis that Fe alleviates Mn-induced inhibition of soredia growth in *H. physodes*.

Aside from Fe, the supply with P is also known to interact with Mn toxicity in vascular plants. The ratio of Mn to P was found to be important for the viability of cotton plants, because Mn reduces the availability of P by the formation of insoluble complexes (LE MARE 1977). In the aquatic plant *Lobelia dortmanna*, the P content is reduced due to encrustations of Fe and Mn on the root surfaces (CHRISTENSEN & SAND-JENSEN 1998). In soredia of *H. physodes*, HAUCK et al. (2002d) found extracellular encrustations, primarily consisting of Mn and P. Furthermore, Mn is intracellularly immobilized in

polyphosphate granules both in the mycobiont and in the photobiont. In the photobiont, this process is accompanied by a significant accumulation of P in the polyphosphate granules. X-ray microanalysis suggests that P required for extra- and intra-cellular Mn immobilization is partly derived from the fungal cell walls. These observations justify the hypothesis that the formation of Mn-P complexes in *H. physodes* could result in P deficiency. The adenine nucleotide concencentrations are sensitive indicators of intracellular P deficiency (OLSEN et al. 1985). Therefore, we tested the hypothesis that Mn uptake in *H. physodes* reduces the ATP content.

2. Materials and methods

2.1. Chlorophyll concentrations

Hypogymnia physodes (L.) Nyl. and *Lecanora conizaeoides* Nyl ex Crombie were sampled in the Göttingen area (51°33' N, 9°57' E) and stored in plastic bags at dark at 5-10 °C for a few days prior to use; both species contain *Trebouxia jamesii* (Hildreth and Ahmadjian) Gärtner as photobiont. Thalli of *H. physodes* were briefly moistened with water, put on paper towels, cleaned from bark and cut into pieces of about 1 cm^2 (i.e. of about 20 mg d. wt.). Thalli of the crustose *L. conizaeoides* were scraped of tree boles with a razor blade; thallus fragments obtained by this method weighed about 10 mg d. wt. The thallus pieces of either species were preincubated on moist filter paper in Petri dishes for one day at 80 % relative humidity, a day temperature (for 13 h daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1}, and a night temperature of 10 °C in the growth chamber. Thallus pieces of either species were gently shaken for 1 h with salt solutions. *H. physodes* was incubated with 25 ml either of 0.1, 0.5 or 1.0 mM $MnCl_2$, whereas *L. conizaeoides* was incubated with higher concentrations because of its higher toxitolerance (25 ml either of 0, 1 or 10 mM $MnCl_2$). As an additional reference, both lichen species were incubated with 1 mM $CuCl_2$, as Cu is known to reduce the chlorophyll content in lichens significantly (CHETTRI et al. 1998). Further, *H. physodes* was incubated alternatively with (1) 1 mM $MnCl_2$ and 1 mM $CaCl_2$, (2) 1 mM $MnCl_2$ and 1 mM $MgCl_2$ and (3) 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Solutions of (1) deionized water, (2) 1 mM $CaCl_2$ and (3) 1 mM $MgCl_2$ served as controls. For investigating interrelations between Fe and Mn in *H. physodes*, thallus pieces were

treated with one of the following solutions: (1) deionized water, (2) 5 mM $MnCl_2$, (3) 5 mM $MnCl_2$ + 250 µM $FeCl_3$, (4) 5 mM $MnCl_2$ + 500 µM $FeCl_3$, (4) 5 mM $MnCl_2$ + 750 µM $FeCl_3$, (5) 5 mM $MnCl_2$ + 1 mM $FeCl_3$, (6) 1 mM $FeCl_3$. All solutions were adjusted to pH 3.1 using HCl and NaOH. Calculation of the chemical activity of Mn in the mixed solutions revealed that more than 99 % was available as free Mn^{2+} in the solutions with Ca and/or Mg, whereas 97 - 98 % was available as free Mn^{2+} in the mixed solutions with Fe. Treatments were run in eight replicate samples, except for the Fe experiment where ten replicates were carried out. Subsequent to incubation, the samples were filtered and the lichen material was stored on moist filter paper in Petri dishes for one day in the growth chamber under climatic conditions as described above. With this day in the growth chamber following the exposure to metal sollutions, medium-term reactions of the lichen thalli could be studied, as this is ecologically more relevant compared to short-term reactions that are reversible within a few hours. A borer was used to cut out three discs of 0.28 cm^2 per replicate (each with a dry weight of about 5 - 6 mg). Thallus pieces were transferred into test tubes with 3 ml *N,N*-dimethyl formamide (DMF), wrapped in aluminum foil, immediately placed in a closed box and stored for 24 h at 5-10 °C at dark (MORAN 1982). In a preliminary test with *H. physodes*, DMF was identified as more effective at extracting chlorophyll than dimethyl sulphoxide (DMSO; RONEN & GALUN 1984). A further preliminary test was conducted in order to detect possible interferences of acidic secondary lichen substances in the pigment measurements, but no significant difference occurred between samples where lichen substances were extracted with acetone before the measurements (BROWN & HOOKER 1977) and samples without acetone treatment. The absorbance of the extracts was determined using a Shimadzu photometer (UV120/02) at λ = 603, 625, 647 and 664 nm. Concentrations of chlorophylls *a* and *b* (Chl *a*, Chl *b*) were calculated according to the following equations, which are particularly suitable for low concentration ranges (MORAN, 1982): Chl *a* [ppm] = 12.81 A_{664} - 2.16 A_{647} - 1.44 A_{625} - 4.91 A_{603}; Chl *b* [ppm] = -4.93 A_{664} - 26.01 A_{647} + 3.74 A_{625} - 15.55 A_{603}. Chlorophyll concentrations were related to lichen dry weight (determined after drying for at least 12 h at 105 °C) by gravimetrically determining the water content of each replicate sample.

2.2. Soredia growth

The growth test with soredia of *H. physodes* was carried out according to HAUCK et al. (2002d). The lichens were collected the day before usage and stored in a plastic bag at ca. 5 °C at dark. Soredia were cultivated in Petri dishes with mineral agar medium. The medium (Bold's basal medium; BBM) contains 2.9 mM $NaNO_3$, 1.3 mM KH_2PO_4, 430 µM K_2HPO_4, 300 µM $MgSO_4$, 220 µM $CaCl_2$, 430 µM NaCl, 185 µM H_3BO_4, 18 µM $FeSO_4$, 31 µM $ZnSO_4$, 7 µM $MnCl_2$, 5 µM MoO_3, 6 µM $CuSO_4$, 2 µM $Co(NO_3)_3$, 130 µM Na_2EDTA, 550 µM KOH, and 18 g/l agar. Prior to the addition of agar the pH was adjusted to 3.1 using HCl and NaOH, which corresponds to the pH found in bark and stemflow of *Picea abies* in the Harz Mountains, northern Germany (HAUCK 2000). $MnCl_2$ and $FeCl_3$ were added to BBM in the following concentrations: (1) 7 mM $MnCl_2$, (2) 7 mM $FeCl_3$, (3) 7 mM $MnCl_2$ + 1 mM $FeCl_3$, (4) 7 mM $MnCl_2$ + 3 mM $FeCl_3$, (5) 7 mM $MnCl_2$ + 5 mM $FeCl_3$, (6) 7 mM $MnCl_2$ + 7 mM $FeCl_3$. Fe^{3+} was preferred over the physiologically more active Fe^{2+}, as most Fe is thought to occur in the oxidized form in water films and on the bark surface of trees (BATES & BROWN 1981). A further treatment was run with 7 mM $MnCl_2$ + 7 mM KCl; this should test the effect of Cl^- on soredia growth. Petri dishes with BBM at pH 3.1 without additives served as controls. About 500 soredia were spread over each Petri dish by removing the soredia from the thalli directly on the agar plate with forceps with pointed tips. The plates were stored in a growth chamber for 8 d at 80 % relative humidity, a day temperature (for 13 h daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1} and a night temperature of 10 °C. The experiment was carried out in five replicates. After incubation small, whitish, not grown soredia and green, larger soredia, where propagation of algal cells took place, were separately counted. Viability of soredia of *H. physodes* varies widely even under optimal growth conditions depending on the season. Therefore, the tests were run in early spring (March) when viability is highest (FIECHTER & HONEGGER 1988). The rates of grown soredia are given as percent of the controls.

2.3. Adenine nucleotide concentrations and adenylate energy charge

H. physodes pieces of 1 cm^2 were preincubated in the growth chamber for one day (cf. Ch. 2.2). Thallus pieces were incubated on a shaker for 1 h with solutions of either 0, 0.5, 1, 3, 7, or 10 mM $MnCl_2$ at pH 3.1. Afterwards thallus pieces were removed by

decantation and with forceps and were briefly put on paper towels to remove free excess water. Then pieces were immediately frozen in liquid nitrogen at -196 °C. Additional samples treated with 0 and 10 mM $MnCl_2$, respectively, were put back into the growth chamber for one recovery day prior to freezing in liquid nitrogen. Each treatment was run in five replicates. The extraction and analysis of adenine nucleotides was conducted according to DYCKMANS & RAUBUCH (1997). Samples were ground in liquid nitrogen with mortar and pestle. About 1 g of the homogenate was stirred with 4 ml DMSO for 2 min, before 16 ml of a buffer solution containing 0.1 M Na_3PO_4 and 0.2 M Na_2-EDTA at pH 12 were then added and stirred for further 2 min. Solutions were then stored for 2 min in an ultrasonic disintegrator bath (47 kHz, 95 W). An aliquot of 500 µl was mixed with 500 µl of the quaternary detergent NRB (Lumac, Landgraaf, The Netherlands). The solution was sonified for 5 s and filtered with 0.45 µm cellulose nitrate membrane filters (Sartorius, Göttingen, Germany) under addition of 2 × 1 ml 0.1 M KH_2PO_4. Chloracetaldehyde (CAA; 200 ml) was added to convert the adenine nucleotides into fluorescent 1-N^6-etheno-adenosine nucleotides (ε-adenylates; KOCHETKOV et al., 1971). The extracts were filled up to 5 ml with 0.1 M KH_2PO_4 and kept for 30 min in a water bath at 85 °C. Samples were analyzed with high-performance liquid chromatography (HPLC) using a 64.00 pump (Knauer, Berlin, Germany), a GINA 50 automatic sample injector and an RF 1001 fluorescence detector (Gynkotek, Germering, Germany). A 250 × 4.6 mm column (5 µm ODS Hypersil, Gynkochrom) with a guard column (10 × 4.6 mm) was used for separating the ε-adenylates. The chromatography was performed isocratically with 50 mM ammonium acetate buffer containing 1 mM EDTA and 0.4 mM tetrabutyl ammonium hydrogensulphate (TBAHS) mixed with methanol (89.5 : 10.5, *v/v*) as mobile phase at 26 °C. Fluorescence emission was measured at $\lambda = 410$ nm with 280 nm as the excitation wavelength. Standard solutions were prepared of AMP-$Na_2 \cdot 6\ H_2O$, ADP-$K_2 \cdot 2\ H_2O$, and ATP-$Na_2 \cdot 3\ H_2O$. Adenylate nucleotide concentrations were related to lichen dry weight by determining the water content of each replicate sample. The adenylate energy charge was calculated according to the equation AEC = ([ATP] + 0.5 [ADP]) / ([AMP] + [ADP] + [ATP]).

The applied method for analysis of adenylate nucleotides by HPLC was developed for the determination of soil microbial biomass and was not previously applied to lichens. An example for a chromatogram is shown in Fig. 7-1. In soils, HPLC was found to give

more accurate results compared to enzymatic analysis with luciferin-luciferase (MARTENS 1992). The luciferin-luciferase system was used in previous analyses of the ATP content in lichens (KARDISH et al. 1987; SILBERSTEIN et al. 1990). Attempts to analyze the ATP concentration in $HClO_4$ extracts of *H. physodes* by the enzymatic convertion of ATP and glucose into glucose-6-phosphate and ADP, by the subsequent convertion of glucose-6-phosphate and $NADP^+$ into 6-phosphogluconate and $NADPH^+$, and by the photometrical determination of the $NADPH^+$ concentration (LOWRY & PASSENEAU 1972) were unsuccessful; this method was used by KÖCK & SCHLEE (1981) in isolated cells of *Trebouxia* spec.

2.4 Statistics

All data are given as arithmetic means ± standard error and were tested for normal distribution with the Shapiro-Wilk test. Samples were tested for significant differences with Duncan's multiple range test. In the data of chlorophyll concentrations in Table 7-2 some outlier values occurred; to exclude them from statistical analysis, in each treatment, the highest and the lowest concentration value was schematically skipped. Hence, statistical analysis (including the calculation of mean values) was carried out with eight instead of ten replicate samples. Statistical analyses were computed with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.). Chemical activity of Mn^{2+} in mixed solutions was calculated with Geochem-PC 2.0 (University of California, Riverside, California, U.S.A.).

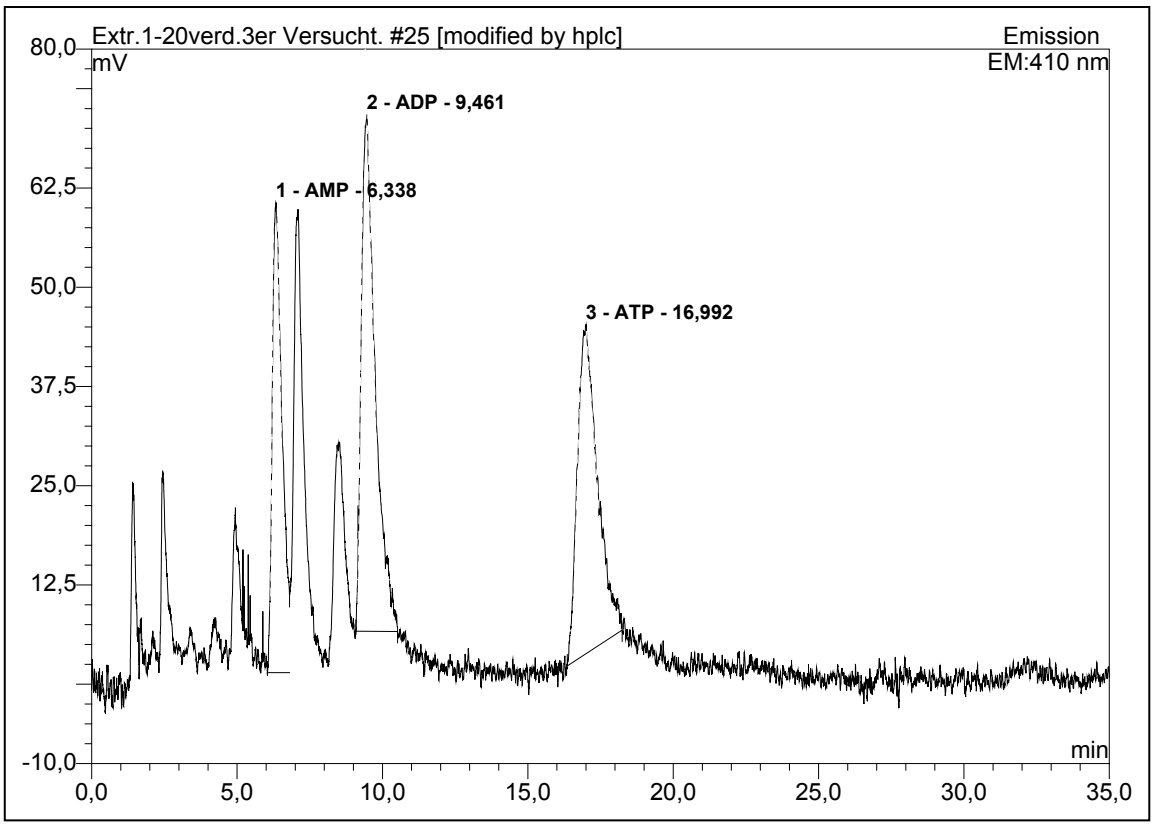

Fig. 7-1. HPLC chromatogram of adenine nucleotides after their derivatization with chloracetaldehyde to fluorescent 1-N^6-etheno-adenosine nucleotides (ε-adenylates). DMSO-extracted *Hypogymnia physodes* sample directly frozen in liquid nitrogen subsequent to incubation with 10 mM $MnCl_2$ for 1 h.

3. Results

3.1. Chlorophyll concentrations

In *Hypogymnia physodes*, chlorophyll concentrations were significantly reduced subsequent to incubation with 1 mM $MnCl_2$ (Table 7-1); Chl *a* content was 58 % and Chl *b* content 61 % of the control. $CuCl_2$ and $MnCl_2$ affected the Chl *a* content equally, whereas the concentration of Chl *b* was less affected by $CuCl_2$ than by $MnCl_2$; at 1 mM $CuCl_2$, the Chl *b* concentration amounted to 92 % of the control. $CaCl_2$ and/or $MgCl_2$

applied together with $MnCl_2$ compensated for Mn-induced reduction of the chlorophyll content.

Table 7-1. Chlorophyll concentrations (in mg g^{-1} d. wt.) in *Hypogymnia physodes* subsequent to 1 h-incubation with metal solutions containing $MnCl_2$, $CaCl_2$, $MgCl_2$, and $CuCl_2$[a]

Treatment [mM]	Chlorophyll *a*	Chlorophyll *b*
Control[b]	2.37 ± 0.04 a	0.38 ± 0.03 a
Ca 1	1.89 ± 0.12 bc	0.34 ± 0.01 ab
Mg 1	2.19 ± 0.13 ab	0.39 ± 0.02 a
Mn 0.1	2.06 ± 0.14 abc	0.34 ± 0.02 ab
Mn 0.5	2.05 ± 0.19 abc	0.29 ± 0.02 bc
Mn 1	1.37 ± 0.09 d	0.23 ± 0.02 c
Mn 1 + Ca 1	2.23 ± 0.17 ab	0.39 ± 0.02 a
Mn 1 + Mg 1	2.02 ± 0.13 abc	0.37 ± 0.03 a
Mn 1 + Ca 1 + Mg 1	1.92 ± 0.15 bc	0.35 ± 0.03 ab
Cu 1	1.74 ± 0.09 cd	0.35 ± 0.01 ab

[a] Arithmetic mean ± standard error. Within a column, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 68$).
[b] Controls run with deionized water.

This was especially true for Chl *b*, the concentration of which was the same as in the control in the assays with $MnCl_2$ and $CaCl_2$ or $MnCl_2$ and $MgCl_2$; it was 92 % of the control in the assay with $MnCl_2$, $CaCl_2$ and $MgCl_2$. Chl *a* was found in concentrations of 94 % ($MnCl_2$ and $CaCl_2$), 85 % ($MnCl_2$ and $MgCl_2$) and 81 % of the control ($MnCl_2$, $CaCl_2$ and $MgCl_2$). Samples incubated with 1 mM $MgCl_2$ (without $MnCl_2$) did not differ from the control with deionized water (Chl *a*: 92 %; Chl *b*: 103 %). 1 mM $CaCl_2$ reduced the Chl *a* content significantly (80 %) and the Chl *b* content insignificantly (89 %). $FeCl_3$ applied in concentrations of up to 1 mM alleviated the reduction of the chlorophyll concentrations induced by 5 mM $MnCl_2$; the Chl *a* and Chl *b* concentrations of the samples treated with combined $MnCl_2/FeCl_3$ solutions were only insignificantly lower than in the control samples (Table 7-2). Chlorophyll concentrations in *Lecanora conizaeoides* were not affected by $MnCl_2$, even though much higher concentrations were applied than in the experiment with *H. physodes* (Table 7-3). $CuCl_2$ reduced the chlorophyll concentrations in *L. conizaeoides*, i.e., to 65 % of the control in the case of Chl *a* and to 84 % in the case of Chl *b*.

Table 7-2. Chlorophyll concentrations (in mg g^{-1} d. wt.) in *Hypogymnia physodes* subsequent to 1 h-incubation with $MnCl_2$ and/or $FeCl_3$[a]

Treatment [mM]	Chlorophyll *a*		Chlorophyll *b*	
Control[b]	2.43 ± 0.14	a	1.36 ± 0.10	a
Mn 5	2.00 ± 0.07	bc	1.04 ± 0.05	b
Mn 5 + Fe 0.25	2.18 ± 0.09	b	1.14 ± 0.05	ab
Mn 5 + Fe 0.50	2.33 ± 0.13	bc	1.12 ± 0.06	ab
Mn 5 + Fe 0.75	2.24 ± 0.14	b	1.16 ± 0.05	ab
Mn 5 + Fe 1	2.30 ± 0.15	bc	1.11 ± 0.06	ab
Fe 1	1.93 ± 0.15	c	0.94 ± 0.06	b

[a] Arithmetic mean ± standard error. Within a column, means sharing a common letter do not differ significantly (Duncan's multiple range test, $p \leq 0.05$, $df = 49$).
[b] Controls run with deionized water.

3.2. Effects of Fe and Mn on soredia growth

In the control samples, 60 ± 6 % of the *H. physodes* soredia started to grow during the 8 d in the growth chamber. The proportion of soredia growing on at 7 mM $MnCl_2$ amounted to 24 % of the control (Fig. 7-2). In the samples, where $FeCl_3$ was applied in combination with 7 mM $MnCl_2$, the rate of grown soredia increased with increasing $FeCl_3$ concentration. In the assay with 3 mM $FeCl_3$ and 7 mM $MnCl_2$, the rate of grown soredia was 64 % of the control. Addition of higher $FeCl_3$ concentrations reduced the viability of the soredia. At 7 mM $FeCl_3$ plus 7 mM $MnCl_2$, the rate of grown soredia (6 % of the control) was significantly lower than at 7 mM $MnCl_2$. Soredia viability at 7 mM $FeCl_3$ was significantly lower than at 7 mM $MnCl_2$. Addition of 7 mM KCl to 7 mM $MnCl_2$ had no effect on soredia growth.

3.3. Effects of Mn on the concentrations of adenosine nucleotides and on the adenylate energy charge

Concentrations of AMP, ADP and ATP in thallus pieces of *H. physodes* were not significantly altered due to 1 h-exposure to $MnCl_2$ concentrations of up to 10 mM (Table 7-4). The day of recovery in the growth chamber after the incubation had no

effect on the adenylate concentrations either. The AEC of the samples treated with Mn tended to be higher than the control without recovery day; these differences were partly significant.

Table 7-3. Chlorophyll concentrations (in mg g^{-1} d. wt.) in *Lecanora conizaeoides* subsequent to 1 h-incubation with metal solutions containing $MnCl_2$ or $CuCl_2$[a]

Treatment [mM]	Chlorophyll *a*	Chlorophyll *b*
Control[b]	2.55 ± 0.08 a	0.62 ± 0.04 a
Mn 1	2.52 ± 0.10 a	0.58 ± 0.02 ab
Mn 10	2.55 ± 0.08 a	0.63 ± 0.03 a
Cu 1	1.65 ± 0.08 b	0.52 ± 0.03 b

[a] Arithmetic mean ± standard error. Within a column, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 26$).
[b] Controls run with deionized water.

4. Discussion

The study shows that Mn affects the chlorophyll concentrations in *H. physodes* not only in the early developing stages (HAUCK et al. 2002d), but also in adult thalli. In *L. conizaeoides*, Mn had no effect on the concentrations of Chl *a* and *b*. Thus, the different effects on the chlorophyll concentrations can be a cause for the different Mn tolerance of these two lichen species. HAUCK et al. (2002c) established that *H. physodes* adsorbs three times more Mn from 1 mM $MnCl_2$ solution during 1 h than *L. conizaeoides*. This difference in Mn adsorption may be a cause for the different effect of Mn on the chlorophyll concentrations. It is remarkable that in the case of Chl *b* $MnCl_2$ was even more detrimental to the chlorophyll concentrations in *H. physodes* than $CuCl_2$. Both Cu^{2+} and Mn^{2+} are borderline ions according to NIEBOER & RICHARDSON (1980), but the former tends more towards the class B ions, whereas the physico-chemical properties of the latter come close to the class A ions.

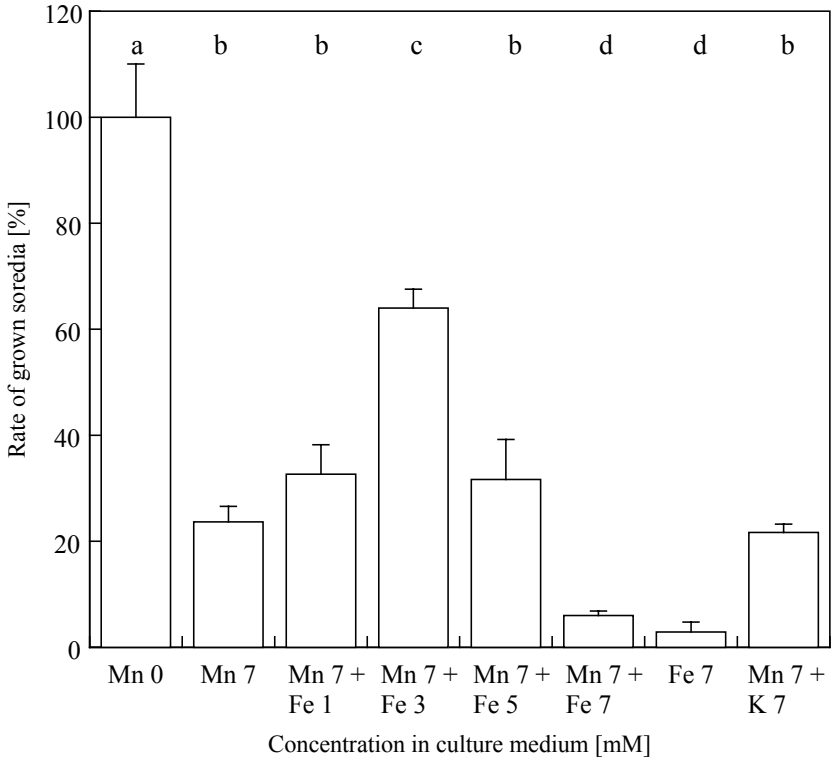

Fig. 7-2. Effects of $FeCl_3$ and KCl on $MnCl_2$-induced inhibition of soredia growth in *Hypogymnia physodes*. Growth rates are specified as percentages of the control. Significant differences are indicated by different letters above the columns (Duncan's multiple range test, $P \leq 0.05$, df = 32). Vertical bars indicate ± SE.

Table 7-4. Concentrations of adenine nucleotides (in pmol mg^{-1} d. wt.) and adenylate energy charge (AEC) in *Hypogymnia physodes* subsequent to 1 h-incubation with $MnCl_2$[a]

Mn [mM]	Rec.[b]	AMP		ADP		ATP		Total[c]		AEC	
Control[d]	-	38.6 ± 3.0	a	68.7 ± 8.8	a	57.2 ± 10.1	a	166 ± 20	ab	0.54 ± 0.03	a
Control[d]	+	33.5 ± 3.3	ab	65.3 ± 7.5	a	65.0 ± 12.4	a	164 ± 20	ab	0.58 ± 0.03	ab
0.5	-	27.2 ± 2.2	ab	56.7 ± 7.6	a	67.5 ± 8.0	a	151 ± 17	ab	0.63 ± 0.01	b
1	-	33.7 ± 3.47	ab	62.2 ± 4.5	a	66.8 ± 5.5	a	163 ± 10	ab	0.60 ± 0.02	ab
3	-	32.6 ± 6.7	ab	59.4 ± 12.4	a	55.9 ± 8.4	a	148 ± 25	ab	0.58 ± 0.03	ab
7	-	21.4 ± 5.2	b	50.3 ± 14.7	a	57.6 ± 17.0	a	129 ± 36	a	0.64 ± 0.02	b
10	-	36.9 ± 6.4	ab	80.8 ± 13.1	a	90.9 ± 9.4	a	209 ± 28	b	0.63 ± 0.01	b
10	+	30.0 ± 6.3	ab	65.3 ± 6.4	a	83.9 ± 10.3	a	179 ± 13	ab	0.65 ± 0.04	b

[a] Arithmetic mean ± standard error. Within a column, means sharing a common letter do not differ significantly (Duncan's multiple range test, $p \leq 0.05$, $df = 29$).
[b] With (+) or without (-) recovery day in the growth chamber subsequent to $MnCl_2$ treatment.
[c] Total of AMP + ADP + ATP concentrations.
[d] Controls run with deionized water.

The grouping into class A, class B and borderline ions specifies the preference of metal ions for different anionic ligands. Class A ions preferably bind to ligands containing O atoms, followed by N and S, whereas class B ions prefer S- over N- as well as N- over O-containing ligands. As the Mg^{2+} ions in the chlorophyll molecule are surrounded by four N atoms, these class A ions are readily replaced by Cu^{2+}. Furthermore, Cu^{2+} has a high affinity for —SH and —S—S— groups and, thus, damages free and membrane-bound proteins (NIEBOER & RICHARDSON 1980). This explains why $CuCl_2$ reduced the concentrations of Chl *a* and *b* in both lichen species. However, the Cu concentration applied is environmentally only relevant on Cu-enriched rocks and in wineyards (PURVIS 1996). TARHANEN (1998) found swollen thylakoid membranes in *Bryoria fuscescens* subsequent to treatment with $CuSO_4$ and $NiSO_4$. Mn^{2+} can also be supposed to damage chlorophyll molecules by displacing Mg^{2+}. Moreover, it apparently binds to membrane-bound ferredoxins in stroma thylakoids (SABNIS et al. 1969, MCQUATTIE & SCHIER 2000). In most studies, which compare the effect of different heavy metals on chlorophyll concentrations or on photosynthesis, Mn is not included. Incubation of the epiphytic lichen *Ramalina lacera* with 20 mM $MnCl_2$ at pH 2.0 for 30 min resulted in a reduction of the total chlorophyll content (as estimated by the ratio of the OD 435 nm/OD 445 nm ratio) to 81 % of unsoaked control samples (GARTY et al. 1992). The chlorophyll content of samples treated with $CuCl_2$ under the same condition amounted to 68 % of the control. However, if the values were related to another control, where samples were treated with H_2O at pH 2.0, the chlorophyll concentrations of the $CuCl_2$ and $MnCl_2$ equalled the control in the former case or were even higher than this control in the case of $MnCl_2$. Ca and Mg reduce the uptake of Mn in *H. physodes* (HAUCK et al. 2002c); this explains the alleviating effect of both elements on Mn-induced chlorophyll degradation. The reduced chlorophyll concentrations in the assay run with $CaCl_2$ alone are in line with reduced growth rates of *H. physodes* soredia cultivated with $CaCl_2$ (HAUCK et al. 2002d).

The alleviating effect of $FeCl_3$ on the Mn-induced reduction of the chlorophyll concentrations suggests that Mn causes Fe deficiency in *H. physodes*. Fe is required for the conversion of protoporphyrin IX into protochlorophyllid in chlorophyll bionsynthesis (HORST 1988, BEALE 1999). Mn-induced accumulation of protoporphyrin IX concomitant to a reduction of chlorophyll concentrations is known from cyanobacteria and vascular plants (CSATORDAY et al. 1984, CLAIRMONT et al. 1986). The positive effect of

low $FeCl_3$ concentrations on chlorophyll concentrations in $MnCl_2$-treated *H. physodes* support the hypothesis that the Mn-induced chlorophyll degradation is, at least in part, due to Fe deficiency. However, the deleterious effect of Mn to chlorophyll concentrations is probably caused by more than one mechanism. It is plausible that, firstly, reduced intracellular Mg/Mn ratios (HAUCK et al. 2002c) and, secondly, the affinity of heavy metals including Mn for ferredoxins bound in stroma thylakoids (SABNIS et al. 1969, HAUCK et al. 2002d, MCQUATTIE & SCHIER 2000) also affect chlorophyll concentrations in *H. physodes*. Fe deficiency probably impaired chlorophyll resynthesis during the recovery day after the incubation with metal solution. The compensation for reduced chlorophyll concentrations due to Fe deficiency is thought to be the main cause for the alleviating effect of $FeCl_3$ in the soredia growth experiment. However, other mechanisms could contribute to growth inhibition of soredia cultivated with excess Mn, as Fe is cofactor of several enzymes such as catalase and cytochrome-C oxidase (HORST 1988).

High $FeCl_3$ concentrations applied alone or in combination with $MnCl_2$ were even more toxic to the soredia than $MnCl_2$. Except for saxicolous species of old mining sites and similar habitats rich in heavy metals, the knowledge of Fe uptake and toxicity in lichens is scarce (HAUCK 2000). In the apoplast, lichens can accumulate significant amounts of Fe without apparent damage. *Cladonia arbuscula* ssp. *mitis* and *Umbilicaria muehlenbergii* adsorbed Fe more effectively than all other metals from mixed solutions containing $CoCl_2$, $CuCl_2$, $FeCl_3$, $NiCl_2$, $PbCl_2$, and $ZnCl_2$ (PUCKETT et al. 1973). KAUPPI & MIKKONEN (1980) measured total Fe concentrations of 800 µmol g^{-1} d. wt. in *H. physodes*, but did not find chlorophyll degradation. Soaking the epiphytic lichen *Ramalina lacera* with either 20 mM $FeCl_3$ or 20 mM $FeCl_2$ at pH 2.0 affected the chlorophyll content less ($FeCl_3$) or equally ($FeCl_2$) compared to treatment with 20 mM $MnCl_2$ (GARTY et al. 1992). The production of stress ethylene was positively correlated with the Fe supply in *H. physodes* and in *R. lacera* (LURIE & GARTY 1991, GARTY et al. 1997). In the terricolous *Nephroma arcticum*, Fe adsorption from 20 mM $FeCl_2$ solution reduced K, Ca and Mg concentrations (GARTY & DELAREA 1991). The results obtained with the agar plates containing 7 mM $MnCl_2$ in combination with 7 mM KCl exhibited that the compensation of $FeCl_3$ for the $MnCl_2$-induced inhibition of soredia growth was not just due to a decreased chemical activity of the Mn^{2+} ions. Furthermore, this assay rules out the possibility that the concentration of Cl^- was decisive for the variation in

soredia viability. This agrees with results of PUNZ (1969), who found net photosynthesis in thalli of *H. physodes* not affected subsequent to exposure to 1 mM NaCl for 18 h.

Mn uptake in *H. physodes* did not impair the formation of ATP by critically reducing the intracellular P level. The immobilization of Mn in polyphosphate granules and extracellular Mn-P crusts (HAUCK et al. 2002d) does apparently not result in P deficiency. The present results parallel results of X-ray microanalyses (combined with transmission electron microscopy) in soredia of *H. physodes* (HAUCK et al. 2002d). The intracellular P concentrations of soredia cultivated with 7 mM $MnCl_2$ did not significantly differ from those in soredia cultivated with 7 µM $MnCl_2$ despite of the presence of intra- and extra-cellular Mn-P deposits in the former. In *H. physodes*, P is effectively taken up from low-concentrated solutions by an active process (FARRAR 1976). This author estimated that contact with rainwater containing 1 µM of inorganic phosphate for 1 h per week would be sufficient for *H. physodes* to maintain its P status. Under field conditions, *H. physodes* can, therefore, be assumed to be capable of quickly compensating for amounts of P consumed for immobilization during Mn uptake. Previous studies of adenylate concentrations in lichens only referred to the ATP content (KARDISH et al. 1987, SILBERSTEIN et al. 1990, 1996). The ATP concentrations detected in *H. physodes* with HPLC and DMSO extraction agree well with ATP concentrations found in samples of *Ramalina lacera* by means of enzymatic analysis subsequent to extraction with either DMSO, $HClO_4$, or Tris combined with EDTA (i.e., 20 - 170 pmol mg^{-1} d. wt.; KARDISH et al., 1987). However, the significance of comparisons of absolute ATP concentrations is limited, as they vary considerably depending on the environmental conditions and the physiological status of the lichen. SILBERSTEIN et al. (1996) repeated an experiment of SILBERSTEIN et al. (1990) to the effect of $NaHSO_3$ on *Ramalina lacera* and *Xanthoria parietina* under the same experimental conditions and found three to four times lower ATP concentrations in all treatments. Moreover, the results strongly depend on the extraction procedure (KARDISH et al. 1987, SILBERSTEIN et al. 1990). The AEC values show that the energy status of *H. physodes* was enhanced in most $MnCl_2$-treated samples. This is seen as an indicator of high metabolic activity due to the $MnCl_2$ exposure. In conclusion the present study shows that Mn-induced chlorophyll degradation in *H. physodes*, but not in *L. conizaeoides* could be a cause for the different Mn sensitivity of these two lichen species as suggested by correlation analyses from field studies in *Picea abies* forests (HAUCK et al. 2001b, 2002a). The

study further showed that the Mn/Ca, Mn/Mg and Mn/Fe ratios were significant for the occurrence of Mn toxicity symptoms in *H. physodes*. The Mn/P ratio is apparently less important. While inverse relationships between the cover of *H. physodes* and other epiphytic lichen species with the Mn/Ca and Mn/Mg ratios in bark or stemflow were repeatedly found by our group in coniferous forests (HAUCK & SPRIBILLE 2002, HAUCK et al. 2002a), no correlations with the Mn/Fe ratio were observed so far. This can be attributed to the loose relationship between total Fe and available Fe in plant tissues. In an unpublished study of our group, only 2 - 3 % of the total Fe content of *Abies balsamea* and *Picea rubens* bark was found to be extractable with water or with 20 mM $SrCl_2$, whereas 35 - 45 % were extracted with EDTA.

Chapter 8

Manganese and its effects on membrane integrity in the epiphytic beard lichens *Alectoria sarmentosa* and *Bryoria fuscescens*

Abstract

The cytoplasma membranes (at least of the mycobiont) in the epiphytic lichens *Alectoria sarmentosa* and *Bryoria fuscescens* are like in the previously studied *H. physodes* not damaged by $MnCl_2$, since intracellular concentrations of K and Mg are not significantly affected. $MnCl_2$ displaces significant amounts of Mg from the extracellular exchange sites. The intracellular ratios of Ca and Mg to Mn decrease, as Mn is increasingly taken up with increasing $MnCl_2$ concentration in the incubation medium. This shift in the element ratio may be a cause for the Mn-sensitivity in *A. sarmentosa*, *B. fuscescens* and other epiphytic lichens such as *H. physodes*.

1. Introduction

Soredia of the foliose epiphytic lichen *Hypogymnia physodes* are susceptible to Mn. In culture experiments on agar plates, the ability of soredia to grow decreased with increasing Mn supply within a concentration range from 7 µM to 10 mM (HAUCK et al. 2002d). This was correlated with decreasing concentrations of chlorophylls *a* and *b*, with structural damage of fungal and algal cells as well as with the immobilization of Mn in intracellular polyphosphate bodies and in extracellular crusts. HAUCK et al. (2002c) showed that Mn is readily taken up in thalli of *H. physodes* from solution. Adsorption of Mn to extracellular exchange sites as well as intracellular uptake are reduced

when Mn was applied in combination with Ca or Mg. This agrees with the observation that Ca and Mg have an alleviating effect on the Mn-induced growth depression in soredia of *H. physodes* (HAUCK et al. 2002d). The crustose lichen *Lecanora conizaeoides* adsorbs less Mn at the extracellular exchange sites than *H. physodes*, whereas intracellular Mn concentrations do not differ between lichen samples exposed to Mn solutions of the same concentration (HAUCK et al. 2002c). In exchange to Mn, *H. physodes* lost extracellularly bound Ca and Mg, whereas intracellular concentrations of Ca and Mg remained unaltered. *L. conizaeoides* lost much smaller amounts of Ca and Mg from the extracellular exchange sites. Further, *L. conizaeoides* had a higher intracellular Mg/Mn ratio than *H. physodes*; the Mg/Mn ratio is known to be decisive for Mn tolerance in vascular plants and yeast (GOSS & CARVALHO 1992, BLACKWELL et al. 1997).

These experimental results agree with correlations found in the field between cover values of lichen species and the Mn concentration in bark and stemflow of *Picea abies*. In spruce stands of the Harz Mountains, northern Germany, cover of *H. physodes* decreased with increasing Mn concentrations and Mn/Ca ratios, whereas cover of *L. conizaeoides* remained unaffected (HAUCK et al. 2001b, HESSE 2002). Not yet published investigations of our group in coniferous forests of western North America revealed negative correlations between the cover of numerous lichen species and the ratio of Mn/Ca concentrations in bark. Since both data from the field and from experiments indicate that Mn can exert a significant influence on the abundance of epiphytic lichens, we conducted further experiments to study mechanisms for Mn sensitivity of epiphytic lichens.

Extra- and intra-cellular K concentrations were not affected in *H. physodes* after 2 h-incubation with 100 mM $MnCl_2$. This indicates the absence of membrane damage, at least, from the mycobiont. The release of intracellular K indicates the loss of membrane integrity, as K is present in high concentrations in the cytoplasm and, further, is a very mobile ion (BROWN & BECKETT 1984, BROWN & BROWN 1991, GARTY et al. 1998). In the present study, we investigated two species of beard lichens (i.e., *Alectoria sarmentosa* and *Bryoria fuscescens*) that are known to be generally less toxitolerant than *H. physodes* (WIRTH 1992). These lichen species are native to coniferous forests both of Germany and North America. In *Picea engelmannii-Abies lasiocarpa* forests of

northwestern Montana, cover of both species (as well as of other *Alectoria* and *Bryoria* species) decreases with increasing Mn/Ca ratio in the substrate. In the present study, the hypothesis was tested that the membranes of these less toxitolerant lichens are injured by Mn. In this experiment, effects on Ca and Mg concentrations were studied in addition to effects on K. Mg that is partly solved in the cytoplasm and partly bound to the cell walls is seen as an indicator of severe membrane damage (BRANQUINHO et al. 1997a). Ca is required for the stabilization of membranes (BROWN & BECKETT 1984).

2. Materials and methods

2.1. Mn uptake and membrane integrity

Alectoria sarmentosa (Ach.) Ach. and *Bryoria fuscescens* (Gyelnik) Brodo & Hawksw. were collected in a boreal *Picea abies* forest of Jämtland, Sweden, and transported in air-dry condition at dark. *A. sarmentosa* and *B. fuscescens* were cut into 1 cm long pieces. Three replicate samples each of about 30 thallus pieces were incubated in either (1) deionized water, (2) 0.1 mM $MnCl_2$, (3) 1 mM $MnCl_2$, or (4) 100 mM $MnCl_2$. Subsequent to incubation, samples were briefly washed with 25 ml deionized water. Lichen samples were stored for one day in the growth chamber (as in HAUCK et al. 2002b) before chemical analysis. Sequential elution was employed for determining the extra- and intra-cellular cation concentrations (BROWN & BROWN 1991). Lichen samples were shaken twice in 30 ml of deionized water for 20 min and twice in 30 ml 20 mM $NiCl_2$ each for 20 min. Then samples were dried at 105 °C, weighed, and digested with 65 % HNO_3 under heat and pressure. The content of K, Mg, Ca, and Mn of all fractions as well as of the incubation media and the water eluate obtained before the recovery day in the growth chamber was analyzed using atomic absorption spectrophotometry (AAS: Varian, SpectrAA 30); Cs^+ was used as a deionizer for the K measurements, whereas K^+ was added for analyzing Ca and La^{3+} in the case of Mg. Element concentrations were related to dry weight. Amounts of cations released from the thallus (fraction *1*) were calculated from the concentrations in the incubation medium and in the water eluate from the washing procedure prior to the recovery day. Concentrations of free ions that occur in the apoplast (*2*) were calculated from the concentrations in the two water eluates obtained after the recovery day. Concentrations of extracellularly bound ions (*3*)

were determined by adding the values from the two $NiCl_2$ extracts. Intracellular concentrations (*4*) were calculated from the HNO_3 digest. Aside from absolute concentrations, percent values were calculated that are related to the total of fractions (*1*) to (*4*).

2.2. Statistics

Data are given as arithmetic means ± standard error. The Shapiro-Wilk test was employed to test data for normal distribution. Samples were tested for significant differences with Duncan's multiple range test. A two-way analysis of variance (ANOVA) was run in order to quantify the effects of the applied $MnCl_2$ concentration, the lichen species, and the interaction between these parameters on element concentrations and ratios in *A. sarmentosa* and *B. fuscescens* (Table 8-2). Statistical significance was tested by calculating *F* values. Statistical analyses were computed with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.). Chemical activity of mixed solutions was calculated with Geochem-PC 2.0 (University of California, Riverside, California, U.S.A.).

3. Results

3.1. Mn uptake and membrane integrity

In the control samples, about 80 % of the Mn in *Alectoria sarmentosa* and *Bryoria fuscescens* were bound to the cell walls (Table 8-1). The intracellular mean Mn concentration amounted to 0.4 µmol g^{-1} d. wt. in *A. sarmentosa* and to 0.6 µmol g^{-1} d. wt. in *B. fuscescens*. Concentrations of extracellularly bound Mn and of intracellular Mn increased with increasing $MnCl_2$ supply. ANOVA revealed that 90 % of the variation of Mn concentration at the extracellular binding sites can be attributed to variations in the $MnCl_2$ content of the incubation medium irrespective of the lichen species (Table 8-2). This relationship was weaker for intracellular Mn concentrations (72 %), as Mn uptake depended in part on the lichen species.

B. fuscescens contained about twice as much intracellular K (35 µmol g^{-1} d. wt.) as *A. sarmentosa* (15 µmol g^{-1} d. wt.; Tables 8-3, 8-4). This difference was highly significant,

whereas the $MnCl_2$ treatment had no decisive effect (Table 8-2). Extracellular K concentrations were negligible with mostly far less than 1 µmol g^{-1} d. wt. In *Alectoria sarmentosa*, treatment with 1 mM $MnCl_2$ caused a slight, but significant increase in the K content of the incubation medium (Fig. 8-1). However, in all $MnCl_2$ treatments, more than 90 % of the total K content remained in the intracellular fraction. In *B. fuscescens*, even 100 mM $MnCl_2$ caused no K efflux into the incubation medium; 99 % of the K was found intracellularly. $CuCl_2$ caused a significant K release from both species. Twenty-five to 30 % were lost to the incubation medium and about 50 % were found in the water-soluble fraction of the apoplast. Only 10 % (*A. sarmentosa*) or 17 % (*B. fuscescens*) remained in the cell interior. The higher efflux of K from *B. fuscescens* compared to *A. sarmentosa* (Fig. 8-1) is in line with the higher absolute intracellular K concentrations of the former species in the control samples.

Table 8-1. Concentrations of extracellularly bound and intracellular Mn (in μmol g^{-1} d. wt.) and ratios to Ca and Mg in *Alectoria sarmentosa* and *Bryoria fuscescens* subsequent to 1 h-incubation with metal solutions containing $MnCl_2$[a]

Incubation [mM][b]	*Alectoria sarmentosa*		*Bryoria fuscescens*	
	Extracellularly bound	Intracellular	Extracellularly bound	Intracellular
Mn content:				
Control	1.36 ± 0.13 a	0.35 ± 0.06 a	2.12 ± 0.29 a	0.63 ± 0.16 a
Mn 0.1	2.60 ± 2.29 a	0.53 ± 0.03 a	2.51 ± 0.08 a	0.90 ± 0.13 a
Mn 1	13.0 ± 2.2 b	1.38 ± 0.15 b	16.5 ± 0.4 b	1.84 ± 0.08 a
Mn 100	49.6 ± 2.4 c	3.88 ± 0.30 c	80.1 ± 5.9 c	9.68 ± 1.24 b
Ca/Mn ratio:				
Control	153 ± 149 a	906 ± 475 a	155 ± 153 a	708 ± 474 a
Mn 0.1	91.5 ± 89.5 a	784 ± 385 a	98.9 ± 95.8 a	514 ± 246 a
Mn 1	17.4 ± 11.1 a	296 ± 113 a	18.7 ± 13.3 a	255 ± 88 a
Mn 100	4.87 ± 4.15 a	94.3 ± 53.1 a	3.83 ± 3.16 a	41.2 ± 19.5 a
Mg/Mn ratio:				
Control	4.48 ± 0.27 a	12.5 ± 4.5 ab	7.59 ± 0.44 a	14.8 ± 2.9 a
Mn 0.1	2.42 ± 0.10 a	18.2 ± 2.7 a	5.61 ± 0.50 a	11.8 ± 1.5 a
Mn 1	0.35 ± 0.07 a	7.25 ± 2.44 bc	0.74 ± 0.06 a	4.39 ± 0.09 a
Mn 100	0.01 ± 0.00 a	1.08 ± 0.15 b	0.02 ± 0.00 a	1.52 ± 0.20 b

[a] Arithmetic means ± standard error. Within a column, separately for Mn concentrations as well as Ca/Mn and Mg/Mn ratios, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 10$).
[b] Controls run with deionized water.

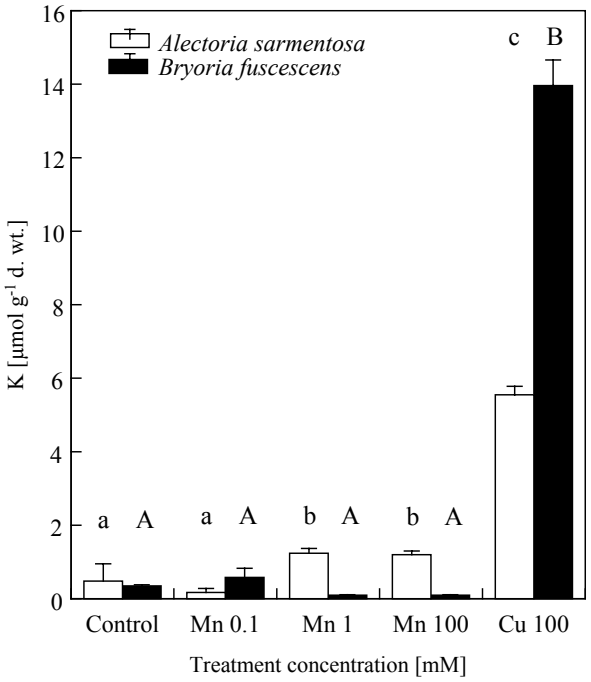

Fig. 8-1. Amounts of K released from *Alectoria sarmentosa*, *Bryoria fuscescens* and *Hypogymnia physodes* during 1 h-incubation to $MnCl_2$ or $CuCl_2$. Within each lichen species, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 8$). Error bars indicate ± standard error.

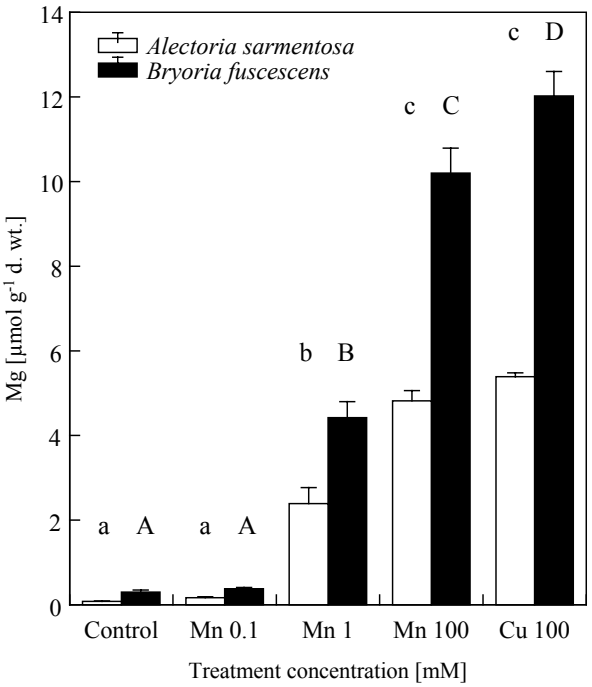

Fig. 8-2. Amounts of Mg released from *Alectoria sarmentosa* and *Bryoria fuscescens* during 1 h-incubation to $MnCl_2$ or $CuCl_2$. Within each lichen species, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 10$). Error bars indicate ± standard error.

Table 8-2. Two-way ANOVA for the effect of the lichen species and of the $MnCl_2$ concentration in the incubation medium on concentrations of K, Ca, Mg and Mn in or released from *Alectoria sarmentosa* and *Bryoria fuscescens*.

Fraction[a]	Total		Species		Mn concentration[b]		Species × Mn	
	Var. [%]	*F* value[c]	Var. [%]	*F* value	Var. [%]	*F* value	Var. [%]	*F* value
K content:								
1	85	13.3 ***	45	49.6 ***	5	1.91	35	12.7 ***
2	52	2.45	28	9.05 **	10	1.14	14	1.56
3	91	23.7 ***	18	33.0 ***	35	21.2 ***	39	23.2 ***
4	82	10.7 ***	63	57.3 ***	4	1.27	15	4.54 *
Ca content:								
1	10	0.24	3	0.54	6	0.34	1	0.04
2	16	0.42	0	0.04	13	0.75	3	0.20
3	3	0.07	1	0.24	0	0.06	0	0.02
4	4	0.09	3	0.49	1	0.04	0	0.00
Mg content:								
1	98	144 ***	9	88.6 ***	80	273 ***	10	34.1 ***
2	82	10.3 ***	12	10.4 **	54	15.8 ***	16	4.79 *
3	98	105 ***	35	265 ***	52	12.9 ***	11	26.4 ***
4	62	3.47 *	19	7.25 *	0	1.95	28	3.72 *
Mn content:								
3	98	144 ***	3	26.1 ***	90	309 ***	5	18.6 ***
4	95	46.8 ***	8	28.0 ***	72	82.5 ***	15	17.3 ***
Ca/Mn ratio:								
3	51	2.34	0	0.06	51	5.22 **	0	0.04
4	51	2.30	1	0.53	34	3.47 *	15	1.58
Mg/Mn ratio:								
3	70	5.23 ***	4	2.72	63	10.7 ***	2	0.42
4	69	4.49 ***	2	1.03	48	7.01 **	19	2.83

[a] *1*: concentration of released or not adsorbed ions; *2*: water-soluble extracellular ions; *3*: extracellularly bound ions; *4*: intracellular ions.
[b] Mn treatments with 0, 0.1, 1, and 100 mM $MnCl_2$.
[c] Levels of significance: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$; $df = 23; 1, 3, 3$.

$MnCl_2$ had no effect on the Ca concentrations in *A. sarmentosa* and *B. fuscescens* (Tables 8-2 – 8-4). About one half of the total amount of Ca was found intracellularly. About 10 - 20 % each were located in the water-soluble and in the $NiCl_2$ exchangeable

extracellular fraction. The intracellular Ca concentrations ranged from 340 to 510 µmol g^{-1} d. wt. Ca concentrations varied considerably in all thallus fractions.

Table 8-3. Concentrations of free apoplastic, extracellularly bound and intracellular K, Ca and Mg (in µmol g^{-1} d. wt.) in *Alectoria sarmentosa* subsequent to 1 h-incubation with metal solutions containing $MnCl_2$ or $CuCl_2$[a]

Incubation [mM][b]	Extracellular, water-soluble	Extracellularly bound	Intracellular
K content:			
Control	0.09 ± 0.28 (1) a	0.01 ± 0.00 (0) a	15.2 ± 1.8 (96) a
Mn 0.1	0.03 ± 0.00 (0) a	0.01 ± 0.00 (0) a	14.4 ± 0.6 (96) a
Mn 1	0.01 ± 0.01 (0) a	0.02 ± 0.01 (0) a	16.5 ± 0.0 (93) a
Mn 100	0.00 ± 0.00 (0) a	0.04 ± 0.01 (0) a	15.2 ± 0.4 (92) a
Cu 100	12.1 ± 0.6 (51) b	1.84 ± 0.35 (8) b	4.03 ± 0.11 (17) c
Ca content:			
Control	155 ± 148 (10) a	174 ± 169 (12) a	342 ± 166 (55) a
Mn 0.1	237 ± 231 (13) a	193 ± 186 (11) a	423 ± 204 (56) a
Mn 1	397 ± 160 (23) a	270 ± 179 (23) a	380 ± 133 (29) a
Mn 100	349 ± 191 (24) a	261 ± 226 (20) a	350 ± 179 (41) a
Cu 100	1.35 ± 0.25 (0) a	45.2 ± 5.3 (4) a	213 ± 5 (17) a
Mg content:			
Control	0.20 ± 0.01 (2) a	6.08 ± 0.47 (64) a	3.41 ± 2.06 (33) a
Mn 0.1	0.18 ± 0.02 (1) a	6.23 ± 0.65 (39) a	9.74 ± 1.99 (59) b
Mn 1	0.28 ± 0.05 (2) a	4.18 ± 0.21 (28) b	9.33 ± 2.61 (55) b
Mn 100	0.54 ± 0.09 (5) b	0.67 ± 0.06 (7) c	4.11 ± 0.31 (41) a
Cu 100	1.19 ± 0.15 (14) c	1.12 ± 0.16 (13) c	0.75 ± 0.3 (9) a

[a] Arithmetic means ± standard error. Numbers in brackets are percent values refering to the total amount of ions in the lichen thallus or released from the lichen thallus. The difference of the total of percent values to 100 % within a row is the percentage of ions released into the incubation medium. Within a column, separately for each element, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 10$).
[b] Controls run with deionized water.

Table 8-4. Concentrations of free apoplastic, extracellularly bound and intracellular K, Ca and Mg (in µmol g^{-1} d. wt.) in *Bryoria fuscescens* subsequent to 1 h-incubation with metal solutions containing $MnCl_2$ or $CuCl_2$[a]

Incubation [mM][b]	Extracellular, water-soluble	Extracellularly bound	Intracellular
K content:			
Control	0.13 ± 0.05 (0) a	0.07 ± 0.04 (0) a	34.9 ± 4.2 (98) a
Mn 0.1	0.52 ± 0.09 (1) a	0.68 ± 0.13 (2) a	32.3 ± 3.0 (95) a
Mn 1	0.13 ± 0.08 (0) a	0.03 ± 0.01 (0) a	30.3 ± 2.0 (99) a
Mn 100	0.36 ± 0.31 (1) a	0.07 ± 0.02 (0) a	37.8 ± 1.9 (99) a
Cu 100	23.2 ± 1.9 (51) b	3.99 ± 0.46 (9) b	4.49 ± 1.08 (10) b
Ca content:			
Control	174 ± 148 (8) a	321 ± 16 (18) a	474 ± 278 (53) a
Mn 0.1	204 ± 193 (10) a	243 ± 234 (11) a	512 ± 246 (56) a
Mn 1	280 ± 187 (13) a	320 ± 232 (15) a	476 ± 168 (53) a
Mn 100	555 ± 308 (29) a	335 ± 281 (19) a	445 ± 255 (40) a
Cu 100	1.91 ± 0.23 (0) a	73.8 ± 19.3 (4) a	325 ± 137 (18) a
Mg content:			
Control	0.37 ± 0.01 (1) a	16.0 ± 0.5 (63) a	8.93 ± 2.06 (34) ab
Mn 0.1	0.21 ± 0.02 (1) a	14.0 ± 1.0 (56) ab	10.3 ± 0.3 (41) ab
Mn 1	0.37 ± 0.03 (1) a	12.2 ± 0.8 (49) b	8.06 ± 0.23 (32) b
Mn 100	1.42 ± 0.35 (7) b	1.19 ± 0.09 (5) c	13.0 ± 2.1 (13) a
Cu 100	2.59 ± 0.03 (13) c	2.21 ± 0.18 (11) c	2.64 ± 1.16 (13) c

[a] Arithmetic means ± standard error. Numbers in brackets are percent values refering to the total amount of ions in the lichen thallus or released from the lichen thallus. The difference of the total of percent values to 100 % within a row is the percentage of ions released into the incubation medium. Within a column, separately for each element, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 10$).
[b] Controls run with deionized water.

$CuCl_2$ released 80 % of the total amount of Ca from the thalli of either lichen species. Mg occurred in the control samples to two thirds at the extracellular bindings sites and to one third within the cells irrespective of the lichen species (Tables 8-4 – 8-6). $MnCl_2$ did not alter the intracellular Mg concentrations, but replaced Mg from the extracellular cation exchange sites; at 100 mM $MnCl_2$, only 5 - 7 % of the total Mg was bound extracellularly. The concentration of water-soluble Mg in the apoplast increased with increasing $MnCl_2$ supply. Exposure to 1 mM $MnCl_2$ and higher increased the Mg content of the incubation medium in both lichen species (Fig. 8-2). At 100 mM $MnCl_2$,

A. sarmentosa lost 50 % of the thallus Mg to the incubation medium, whereas *B. fuscescens* released 75 % Mg. $CuCl_2$ and $MnCl_2$ had the same effect on the concentrations of extracellularly bound Mg, whereas the intracellular Mg concentrations were only reduced by $CuCl_2$. In *A. sarmentosa*, $MnCl_2$ and $CuCl_2$ released similar amounts of Mg concentrations from the thallus into the incubation medium; in *B. fuscescens*, $CuCl_2$ increased the Mg concentration in the incubation medium more than $MnCl_2$ (Fig. 8-2).

The ratios of extracellularly bound and of intracellular Ca to Mn as well as of Mg to Mn decreased with increasing $MnCl_2$ supply (Table 8-1). Two-way ANOVA revealed that this decrease was significant in all cases (Table8-2), whereas Duncan's multiple range test yielded a statistical significance for this decrease only for the intracellular Mg/Mn ratios (Table 8-1).

4. Discussion

Mn concentrations taken up by *B. fuscescens* are similar to those taken up by *H. physodes* (HAUCK et al. 2002c). However, with increasing $MnCl_2$ concentration in the incubation medium, the intracellular Mn concentrations increase faster in *B. fuscescens*, whereas the concentration of extracellularly bound Mn increases faster in *H. physodes*. In *H. physodes* incubated for 1 h, the intracellular Mn content amounted to 0.6 µmol g^{-1} d. wt. in the control samples, to 0.7 µmol g^{-1} d. wt. at 1 mM $MnCl_2$ and to 1.6 µmol g^{-1} d. wt. at 100 mM $MnCl_2$. In *B. fuscescens*, the corresponding concentrations were 0.6 µmol g^{-1} d. wt. (control), 1.8 µmol g^{-1} d. wt. (1 mM $MnCl_2$) and 9.7 µmol g^{-1} d. wt. (100 mM $MnCl_2$). Concentrations of extracellularly bound Mn was 1.6 µmol g^{-1} d. wt. (control), 33 µmol g^{-1} d. wt. (1 mM $MnCl_2$), and 98 µmol g^{-1} d. wt. in *H. physodes* and 2.1 µmol g^{-1} d. wt. (control), 17 µmol g^{-1} d. wt. (1 mM $MnCl_2$), and 80 µmol g^{-1} d. wt. (100 mM $MnCl_2$) in *B. fuscescens*. The faster increase in the extracellularly bound Mn concentration in *H. physodes* may be due to a higher amount of anionic exchange sites than in *B. fuscescens* (MODENESI & VANZO 1986) and may be the cause for the slower increase of the intracellular Mn concentration in the former species, as the extracellular exchange sites serve as a buffer between the cytoplasm and the environment (BROWN 1991). *A. sarmentosa* adsorbed less Mn from 100 mM $MnCl_2$ solution compared to *B. fuscescens* and *H. physodes*. Pertaining to the lower Mn concentrations in the control

samples of *A. sarmentosa*, the considerable variation has to be taken into account that occurs in the metal concentrations within a species, even in thalli from the same site (BUCK & BROWN 1979, GARTY et al. 1996). Thus, it is not legitimate to conclude that *A. sarmentosa* contains generally less Mn compared to *B. fuscescens* and *H. physodes*, until replicate samples from different sites are studied.

Like in *H. physodes* (HAUCK et al. 2002c), $MnCl_2$ has no significant effect on intra- and extra-cellular K concentrations in *A. sarmentosa* and *B. fuscescens*. However, in *A. sarmentosa*, the K concentration in the incubation medium increased slightly, but significantly during exposure to 1 mM $MnCl_2$. The results indicate that the membranes of all three lichen species are not severely injured by Mn. This is in line with findings that incubation of the green-algal lichen *Cladonia rangiferina* did not cause any significant K efflux at $MnCl_2$ concentrations below 1 M (BURTON et al. 1981). However, it has to be pointed out that the applied sequential extraction procedure cannot reliably detect membrane damage that is limited to the photobiont, as the mycobiont would blur such an effect, because it forms the major part of the lichen thallus (NIEBOER et al. 1979, GOWARD & SEAWARD 1981). Moreover, K released from the photobiont would partly be adsorbed by the mycobiont. The drastic loss of intracellular K from *A. sarmentosa* and *B. fuscescens* due to incubation with $CuCl_2$ agrees well with other studies to the effects of Cu on lichens (PUCKETT 1976, NIEBOER et al. 1979, BRANQUINHO et al. 1997a). It is explained by the above-mentioned affinity for S-containing proteins. TARHANEN et al. (1999) also found K loss from *B. fuscescens*, when the lichen was exposed to mixed solutions of $CuSO_4$ and $NiSO_4$. Furthermore, the increasing K release was correlated with decreasing ergosterol concentrations.

In *A. sarmentosa* and in *B. fuscescens*, $MnCl_2$ reduced the concentrations of extracellularly bound Mg significantly. This can be attributed to a displacement by Mn at the cation exchange sites, as the Mn concentrations increased simultaneously. A portion of the released Mg remains in the water-soluble extracellular fraction, whereas most of it was lost to the incubation medium. A significant loss of Mg from the extracellular exchange sites was also observed in *H. physodes*, but not in the Mn-resistent *L. conizaeoides* (HAUCK et al. 2002c). The intracellular Mg concentrations remained unaltered; thus, the increasing Mg concentrations in the incubation medium do not indicate membrane damage. However, the Mg/Mn ratios decreased with

increasing $MnCl_2$ supply because of the increasing intracellular Mn concentrations. This shift of the Mg/Mn ratio could impair the cells, as Mn is supposed to compete with Mg for binding sites (GOSS & CARVALHO 1992, BLACKWELL et al. 1997). Mg is essential for the stabilization of cell structures and of important biomolecules such as DNA, RNA and ATP (NIEBOER & RICHARDSON 1980). Further, the decrease of the Mg/Mn ratio may be the cause for the reduced chlorophyll concentrations in *H. physodes* (HAUCK et al. 2002 c). In contrast to the $MnCl_2$ treatments, the efflux of Mg into the incubation medium during soaking with $CuCl_2$ reflects severe membrane damage, as this process was accompanied by significant loss of Mg from the intracellular space. In *Ramalina fastigiata* and *Usnea* spec., $CuSO_4$ applied for 2 h at concentrations up to 16 mM had no effect on intracellular Mg concentrations (BRANQUINHO et al. 1997a).

Ca is characterized by a considerable variation of both extracellular and intracellular concentrations. Such variation was not found in thalli of *H. physodes* and *L. conizaeoides*, but in soredia of the former species (HAUCK et al. 2002c, d). The variability of Ca concentrations may depend on the lichen species and on the stage of development. BRANQUINHO et al. (1997a) found higher variation in Ca concentrations in *Usnea* spec. than in *Ramalina fastigiata*. In samples incubated with $PbNO_3$, Ca concentrations varied considerably in *Flavoparmelia caperata*, but not in *Lobaria pulmonaria*, *Peltigera canina* and *Ramalina farinacea* (BRANQUINHO et al. 1997b). A possible cause for these differently varying Ca concentrations may be the localization of Ca in oxalate crystals in many lichen species (MODENESI et al. 1997, 2001). The microdistribution of Ca within the lichen thalli should be studied with electron microscopy and X-ray microanalysis to test this hypothesis. SCHADE (1970, 1975) found a considerable inter-and intra-specific variation of the amounts of Ca oxalate crystals in *Alectoriaceae* and *Parmeliaceae* species. Although the Ca concentrations in *A. sarmentosa* and *B. fuscescens* did not depend on the $MnCl_2$ supply, the ratios of Ca to Mn decreased due to the increase of extra- and intra-cellular Mn concentrations. This may be deleterious to both species, as Ca is not only essential for stabilizing cell walls and membranes, but also as a second messenger in the intracellular signal transduction (MALHÓ 1999). In thalli of *H. physodes*, the concentrations of extracellularly bound Ca decreased with increasing $MnCl_2$ supply, whereas intracellular Ca concentration remained unchanged as detected by sequential extraction and AAS (HAUCK et al. 2002 b). X-ray microanalysis combined with transmission electron microscopy revealed a

significant loss of Ca from the cell lumina and the cell walls of the mycobiont in soredia of *H. physodes* cultivated with $MnCl_2$ (HAUCK et al. 2002 d). The insignificant trend for lower extracellular Ca concentrations in $CuCl_2$-treated samples of *A. sarmentosa* and *B. fuscescens* can be attributed to high affinity of Cu for extracellular binding sites (BRANQUINHO et al. 1997a).

In conclusion, $MnCl_2$ is apparently not effective at disrupting cytoplasma membranes in lichens, as the intracellular concentrations of K and Mg were neither affected in *A. sarmentosa* or *B. fuscescens* investigated in the present study nor in *H. physodes* or *L. conizaeoides* studied by HAUCK et al. (2002 c). However, with the applied sequential washing procedure for cation extraction, membrane damage restricted to the photobiont cannot reliably be detected. Decreasing ratios of intracellular Ca and Mg to Mn with increasing $MnCl_2$ supply could damage *A. sarmentosa*, *B. fuscescens* and *H. physodes*. The intracellular Mg/Mn ratio as well as the affinity of heavy metals for ferredoxins in the stroma thylakoids could be a direct cause for the reduced concentrations in $MnCl_2$-treated thalli and soredia (HAUCK et al. 2002d) of *H. physodes*. A higher Mg/Mn ratio in *L. conizaeoides* compared to the other species (HAUCK et al. 2002c) could be responsible for the lack of $MnCl_2$-induced reduction of the chlorophyll concentrations in this lichen. The experimental results agree with decreasing cover values of *A. sarmentosa*, *B. fuscescens* and *H. physodes* found in field studies with Mn concentrations and the ratios of Mn to Ca and Mg, respectively (HAUCK et al. 2001b, HESSE 2002).

Chapter 9

Uptake and toxicity of manganese in epiphytic cyanolichens

Abstract

Mn uptake from $MnCl_2$ solution and chlorophyll fluorescence (as a selected vitality parameter) were studied in the epiphytic lichens *Lobaria pulmonaria* (tripartite, heteromerous lichen with the green alga *Dictyochloropsis* as primary photobiont and *Nostoc* in cephalodia), *Nephroma helveticum* (bipartite, heteromerous lichen with *Nostoc* photobiont) and *Leptogium saturninum* (bipartite, homoiomerous lichen with *Nostoc* photobiont). Extracellular adsorption and intracellular uptake of Mn increased in the order *L. pulmonaria* < *N. helveticum* < *L. saturninum*. Mn increasingly reduced the effective quantum yield of photosystem 2 (Φ_2) in the same order. $CaCl_2$ and $MgCl_2$ alleviated the Mn-induced reduction of Φ_2. Moist thalli of all species transferred significant amounts of extracellular Mn into the cells during a recovery day subsequent to incubation with metal solution. This suggests that even short exposures to Mn in the field, e.g. via stemflow, can affect the physiology of the lichen species studied. The experimental results support the hypothesis that cyanolichens are sensitive to excess Mn. Data also suggest that the tripartite *L. pulmonaria* is less Mn-sensitive than the bi-partite cyanolichens. This agrees with published field observations from Montana, where bipartite cyanolichens (including *L. saturninum* and *N. helveticum*) occurred on conifer bark with the lowest Mn concentration, while *L. pulmonaria* was also found on bark with higher Mn concentrations.

1. Introduction

Experimental evidence from the foliose epiphytic lichen *Hypogymnia physodes* shows that this lichen species is sensitive to excess Mn, e.g., in terms of chlorophyll content, chloroplast integrity, soredia growth, photobiont reproduction or in respect of the Ca and Mg budgets (HAUCK et al. 2002c, d, 2003, PAUL et al. 2003, 2004). Correlations between cover of *H. physodes* and Mn concentration in bark or stemflow suggest that the experimentally proven Mn sensitivity limits the abundance of *H. physodes* in the field. So far, such evidence is limited to coniferous forests of Europe and North America (HAUCK et al. 2001, 2002a, SCHMULL & HAUCK 2003a). Mn affecting epiphytes on the trunk surfaces is primarily soil-borne. It reaches the bark surface after root uptake, xylem transport and subsequent leaching from bark and foliage (LÖVESTAM et al. 1990, SLOOF & WOLTERBEEK 1993, LEVIA & HERWITZ 2000). In the sites studied, so far, in Germany, New York and Montana, Mn was, therefore, a natural site factor and did not derive primarily from atmospheric deposition (HAUCK 2003, 2005). Whether Mn is effective only in conifer stands, with acidic soils, bark, stemflow and throughfall, where the availability of Mn is higher than at less acidic sites, or whether Mn toxicity is a widespread site factor for epiphytic lichens even in deciduous forest ecosystems is not known yet. Further, it is not known whether high ambient Mn concentrations limit the abundance of epiphytes other than lichens. In contrast to *H. physodes*, the crustose lichen *Lecanora conizaeoides* is not sensitive to Mn as shown by field data (HAUCK et al. 2001b, 2002a) and experimental evidence (HAUCK et al. 2002c, 2003). This is due to its effective intracellular immobilization in polyphosphate granules and in S-containing deposits, which may be phytochelatines (PAUL et al. 2003).

As yet, investigations on Mn toxicity have been limited to chlorolichens with the most common green-algal photobiont *Trebouxia*. Particularly with regard to SO_2 or heavy metals, cyanolichens are often supposed to be less toxitolerant than chlorolichens (GARTY 2001, NASH & GRIES 2002). In northwestern Montana, cyanolichens such as *Leptogium cellulosum*, *L. saturninum*, *Lobaria hallii*, *Nephroma helveticum*, *N. parile*, and *N. resupinatum* were found to be restricted to coniferous tree bark with low Mn content (HAUCK & SPRIBILLE 2002). Bark with high Mn content was only inhabited by chlorolichens. The tripartite lichen *Lobaria pulmonaria*, which has a green alga as the primary photobiont and the cyanobacterium *Nostoc* in internal cephalodia, had an

intermediate position between bipartite cyano- and chloro-lichens with respect to the Mn concentration of the substrate.

These field observations from Montana led to the hypothesis that cyanolichens are particularly sensitive to Mn. Furthermore, the observation with *Lobaria pulmonaria* resulted in the hypothesis that bipartite cyanolichens are more sensitive to Mn than tri-partite lichens with cephalodia. To test these hypotheses, three species were selected that occur on conifers of northwestern Montana (HAUCK & SPRIBILLE 2002) and represent three different types of cyanolichens. *Leptogium saturninum* is an homoiomerous, gelatinous, bipartite cyanolichen, *Nephroma helveticum* is an heteromerous, bipartite cyanolichen lichen, and *Lobaria pulmonaria* is an example of an heteromerous, tripartite lichen. All species contain the cyanobacterium *Nostoc*; the green-algal photobiont of *L. pulmonaria* is *Dictyochloropsis* (RIKKINEN 2002). To test the effect of Mn of these lichen species, we studied intra- and extra-cellular uptake as well as chlorophyll fluorescence. Since, firstly, field observations in Montana indicated that the ratios of Mn to Ca and Mg could be more significant for cyanolichens distribution than the Mn concentration itself (HAUCK & SPRIBILLE 2002), and secondly, experimental and field evidence for interaction of Mn with Ca and Mg is available from the chlorolichen *Hypogymnia physodes* (HAUCK et al. 2002a, c, d, 2003), Mn uptake and chlorophyll fluorescence were also studied in assays where Mn was combined with Ca or Mg.

2. Materials and methods

2.1. In vitro Mn uptake

Thalli of *Leptogium saturninum* (Dickson) Nyl., *Lobaria pulmonaria* (L.) Hoffm. and *Nephroma helveticum* Ach. were sampled from conifer bark in British Columbia, Canada. Samples of *L. saturninum* and *N. helveticum* were taken in Wells-Gray Provincial Park, ca. 25 km N Clearwater, 51°49' N, 120°07' W, 820 m, those of *L. pulmonaria* in Spahats Provincial Park, N Clearwater, 51°45' N, 120°00' W, 760 m. Air-dry thalli were stored in the dark at room temperature for a few days subsequent to collection and during transport by aircraft and were then frozen at -30 °C. For uptake experiments, thalli were cut into pieces of about 1 cm^2 at room temperature. These pieces were mixed (separately for each species) to avoid effects due to variation of vitality or element content between different thalli of the same species. Prior to the experiment, samples were stored in Petri dishes for one day at 80 % relative humidity, a day temperature (for 13 hours daily) of 13 °C during a photon flux of 30 µmol m^{-2} s^{-1}, and a night temperature of 10 °C. Experiments were carried out in five replicates, while each replicate sample consisted of ten thallus pieces. *L. pulmonaria* and *N. helveticum* were incubated in 20 ml of 5 mM $MnCl_2$ for 0, 2.5, 5, 10, 20, 40, or 80 min, respectively, in order to study time-dependent Mn uptake. The effect of Ca and Mg on Mn uptake was studied in *L. pulmonaria*, *N. helveticum* and *L. saturninum*. Samples were incubated in 20 ml either of 10 mM $MnCl_2$, 10 mM $MnCl_2$ and 2.5 mM $CaCl_2$, or 10 mM $MnCl_2$ and 2.5 mM $MgCl_2$ for 40 min. *L. saturninum* was only considered in this part of the experiment, because it was not possible to collect enough material of this species at one site for the entire experiment. All solutions were adjusted to pH 5 with HCl and NaOH. Incubation was stopped by removing the incubation solution by decantation, immediately shaking the samples with 20 ml deionized water for 2 min and subsequent removal of the water. After incubation, one half of the samples was stored in the growth chamber under the climatic conditions as described above for one day. By this recovery day, lagged uptake of Mn, i.e., translocation from extracellular binding sites into the cell was studied. The other half of samples was prepared for analysis immediately after incubation.

For this purpose, samples were shaken twice with 20 ml of deionized water to remove free apoplastic ions. These water samples were not analyzed, because Mn is primarily allocated at extracellular exchange sites and intracellularly. Extracellularly bound cations were exchanged by shaking samples twice with 20 ml $NiCl_2$. The two $NiCl_2$ solutions per sample were filtered with ash-free filters (Blue Ribbon Filters, Schleicher & Schuell, Dassel, Germany) and pooled. According to VÁZQUEZ et al. (1999) two washing procedures with 20 mM $NiCl_2$ are sufficient to release extracellularly bound ions of class A metals or borderline ions with class A character (NIEBOER & RICHARDSON 1980), whereas $NiCl_2$ incubation with higher concentrations or for prolonged periods results in membrane damage. Then samples were dried at 105 °C, homogenized, and digested with 65 % HNO_3 in order to determine the intracellular ions (BROWN & BROWN 1991). Concentrations of Mn, Ca and Mg in $NiCl_2$ solutions and of Mn, Ca, Mg, and K in acid digests were determined with AAS (AAS Vario 6, Analytik Jena, Germany); 0.1 % $CsCl_2$ and 0.1 % $La(NO_3)_3$ were added prior to analysis to suppress ionization of K or the to release Ca and Mg from refractory, insoluble salts, respectively.

2.2. Chlorophyll fluorescence

Thallus lobes of *L. pulmonaria*, *N. helveticum* and *L. saturninum* were preincubated in the growth chamber, incubated with either (1) deionized water (control), (2) 5 mM $MnCl_2$, (3) 10 mM $MnCl_2$, (4) 5 mM $MnCl_2$ and 2.5 mM $CaCl_2$, (5) 5 mM $MnCl_2$ and 2.5 mM $MgCl_2$, or (4) 5 mM $MnCl_2$, 1.25 mM $CaCl_2$ and 1.25 mM $MgCl_2$ at pH 5 for 40 min as described above. Afterwards the incubation solution was removed by decantation, samples were shaken with 20 ml deionized water for 2 min and the water was removed. Lichens were stored in the growth chamber under climatic conditions as described in Ch. 2.1. for two weeks.

Chlorophyll fluorescence was measured with a pulse amplitude modulated fluorimeter (Mini-PAM Photosynthesis Yield Analyzer, Walz Mess- und Regeltechnik, Effeltrich, Germany). Measurements were carried out one day before the incubation with metal solutions (day 1) as well as two days (day 3) or 14 days (day 15) after the incubation. All measurements were conducted in the growth chamber at a photon flux of 30 µmol

m^{-2} s^{-1}. The samples were exposed to a weak measuring beam (0.15 mmol photons m^{-2} s^{-1}, modulated at 0.6 kHz from a light-emitting diode with a peak emission at λ = 655 nm). Steady state fluorescence yield (F_S) was taken as the average of this signal during the first 5 s. A saturation pulse of high-intensity white light (8000 mmol m^{-2} s^{-1} for 0.8 s) was then applied to produce full closure of the photosystem 2 (PS2) photochemical reaction centers, i.e., the maximum fluorescence level in the light adapted state (F'_M). From this data, the effective quantum yield of photochemical energy conversion in PS2 ($\Phi_2 = F'_M–F_S/F'_M$) was calculated (ROHÁCEK 2002).

2.3. Statistics

All data are given as arithmetic means ± standard error and were tested for normal distribution with the Shapiro-Wilk test. Samples were tested for significant differences with Duncan's multiple range test. An analysis of variance (ANOVA) was carried out in order to quantify to what extent the intra- and extra-cellular ion concentrations and ratios depended on lichen species, incubation time and presence or absence of a recovery day after metal exposure (Table 9-2). Statistical analyses were computed with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.). Michaelis-Menten regression lines for intra- and extra-cellular Mn uptake in Figs. 9-1 – 9-4 were calculated with the program Xact 4.01, SciLab Co., Hamburg.

3. Results

3.1. In vitro Mn uptake

Intracellular Mn uptake strongly increased in the order *L. pulmonaria* < *N. helveticum* < *L. saturninum* (Table 9-1). Mn uptake versus time was studied in *L. pulmonaria* and *N. helveticum* (Fig. 9-1, Table 9-2). While *L. pulmonaria* took up about 1.6 µmol Mn per g dry weight after 80 min, uptake in *N. helveticum* was twice as much at 3.1 µmol g^{-1} d. wt. Intracellular uptake reached saturation within a few minutes in both species (Fig. 9-1).

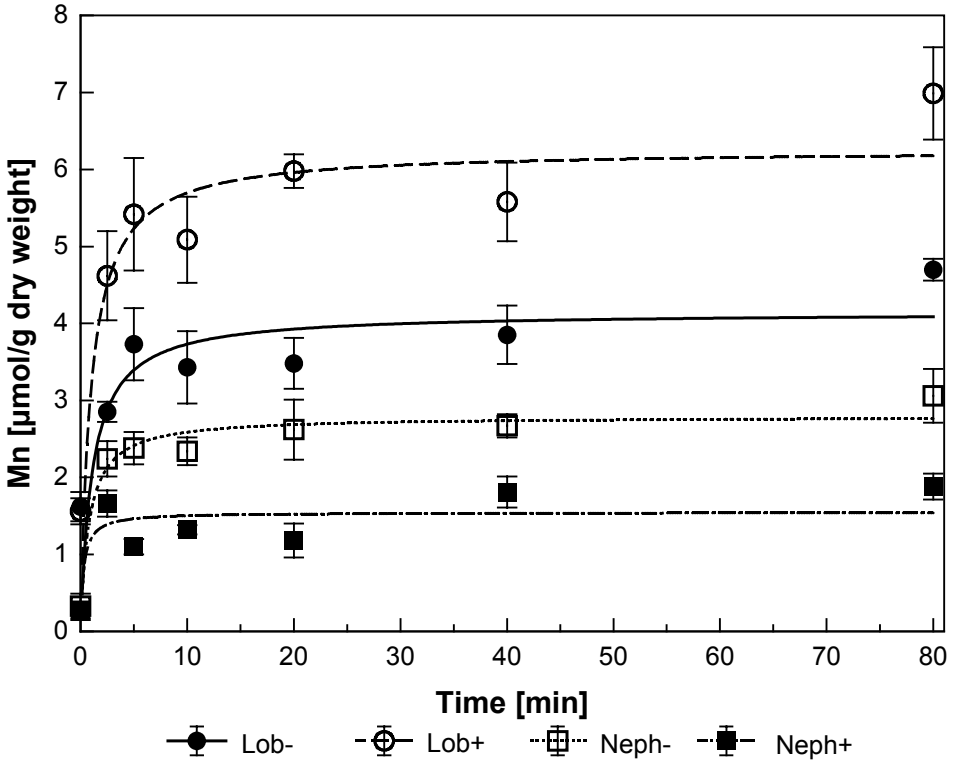

Fig. 9-1. Intracellular Mn uptake in *Lobaria pulmonaria* and *Nephroma helveticum* related to time from 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob –, Neph –) or after a subsequent recovery day in the growth chamber (Lob +, Neph +). Arithmetic means of five replicates ± standard error.

One day of recovery in the growth chamber after incubation with Mn, resulted in intracellular Mn concentrations that were increased by 75 ± 13 % in *L. pulmonaria* and 51 ± 4 % in *N. helveticum*. The species-specific difference between these means (calculated

from samples that were incubated with Mn alone) was statistically significant ($P \leq 0.05$, *t*-test, df = 6). In *L. saturninum*, one day of recovery increased the intracellular Mn content by 26 % in samples incubated with 10 mM $MnCl_2$ for 40 min. Samples that did not reach the intracellular saturation level of Mn immediately after incubation because of too short incubation time, had still lower intracellular Mn content after the recovery day than samples, which were exposed more extendedly to $MnCl_2$ and, thus, reached intracellular saturation immediately. This is remarkable, as extracellular exchange sites exhibited an Mn content that was about ten times as high as in the cell interior (Fig. 9-2, Table 9-3). Extracellular adsorption of Mn did not differ between *L. pulmonaria* and *N. helveticum* immediately after incubation. However, after the recovery day, adsorption was lower in *L. pulmonaria* than in *N. helveticum*. In the former, 13.2 ± 2.0 μmol g^{-1} Mn d. wt., and in the latter 8.6 ± 1.1 μmol g^{-1} Mn d. wt. were removed from the extracellular cation exchange sites. In *L. saturninum*, 3.2 μmol g^{-1} Mn d. wt. were removed from the exchange sites in samples incubated with $MnCl_2$. Intracellular Mn levels of control samples as collected from the field were higher in *L. saturninum* and *N. helveticum* than in *L. pulmonaria* (Table 9-1), while extracellularly bound Mn concentrations were not different (Table 9-3).

At the extracellular exchange sites, Mn uptake strongly reduced concentrations of Ca (Fig. 9-3) and Mg (Fig. 9-4). In all species, extracellular Ca and Mg content increased during the recovery day (Figs. 9-3, 9-4; Table 9-3). Mn uptake had no significant effect on intracellular concentrations of K, Ca and Mg (Tables 9-1, 9-2). Thus, intracellular Ca/Mn and Mg/Mn ratios decreased only because of increased Mn concentrations (Tables 9-1, 9-2). Incubation with $MnCl_2$ in combination with $CaCl_2$ or $MgCl_2$ did not reduce intracellular Mn concentrations in *N. helveticum* and *L. saturninum*, but in *L. pulmonaria* (Table 9-1). Concentration of Mn at extracellular exchange sites was reduced by, respectively, $CaCl_2$ and $MgCl_2$ in all species, however, in *L. saturninum* only after the recovery day (Table 9-3).

Table 9-1. Intracellular concentrations of Mn, Ca, Mg, K (in μmol g^{-1} d. wt.) and molar ratios of Ca and Mg to Mn in *Lobaria pulmonaria*, *Nephroma helveticum* and *Leptogium saturninum* after treatment with 10 mM $MnCl_2$ alone or in combination with 2.5 mM $CaCl_2$ or 2.5 mM $MgCl_2$ for 40 min

	Rec.[a]	Control		Mn		Mn + Ca		Mn + Mg	
Lobaria pulmonaria:									
Mn	–	0.27±0.12	a	1.95±0.20	b	1.07±0.13	c	1.19±0.07	c
	+	0.33±0.16	a	3.14±0.25	b	1.29±0.08	c	1.61±0.24	c
Ca	–	7.84±3.01	a	2.01±1.22	a	4.48±2.12	a	3.71±2.91	a
	+	4.16±2.80	ab	0.96±0.44	a	4.98±1.81	ab	7.72±1.99	b
Mg	–	17.4±1.8	ab	19.3±0.77	ab	16.9±1.4	a	21.2±0.84	b
	+	17.6±1.3	a	16.5±1.3	a	19.1±1.3	a	17.7±1.93	a
K	–	127±9	a	131±8	a	108±14	a	137±11	a
	+	130±8	a	120±8	a	123±5	a	123±13	a
Ca/Mn	–	37.1±17.5	a	0.94±0.50	b	4.44±2.25	b	2.87±2.21	b
	+	37.5±20.2	a	0.30±0.13	b	3.76±1.39	b	4.76±1.08	b
Mg/Mn	–	70.6±20.6	a	10.4±1.2	b	16.6±2.1	b	18.0±0.97	b
	+	125±49	a	5.25±0.11	b	15.2±1.7	b	11.5±1.49	b
Nephroma helveticum:									
Mn	–	1.62±0.19	a	4.18±0.55	b	3.94±0.40	b	3.92±0.49	b
	+	1.56±0.17	a	5.76±0.48	bc	6.17±0.24	b	4.33±0.63	c
Ca	–	6.15±2.02	a	13.6±1.7	a	40.5±76.3	a	3.94±1.08	a
	+	4.97±2.19	a	6.83±2.67	a	3.01±1.65	a	2.30±1.43	a
Mg	–	15.5±2.0	a	19.5±2.1	a	18.9±1.48	a	20.1±1.3	a
	+	18.1±2.5	a	18.9±2.4	a	18.2±0.95	a	15.0±1.2	a
K	–	148±8	a	213±20	b	136±10	a	142±14	a
	+	166±18	ab	198±21	a	136±4	b	127±8	b
Ca/Mn	–	3.87±1.25	a	3.28±0.30	a	12.9±11.3	a	0.96±0.28	a
	+	2.61±1.63	a	0.83±0.33	a	0.33±0.25	a	0.29±0.26	a
Mg/Mn	–	9.31±1.82	a	4.80±0.54	b	4.92±0.50	b	5.32±0.47	b
	+	10.5±1.3	a	3.01±0.37	b	2.85±0.16	b	3.74±0.50	b
Leptogium saturninum:									
Mn	–	1.51±0.18	a	12.5±1.1	b	12.4±1.1	b	10.3±0.6	b
	+	2.32±0.38	a	15.7±0.9	b	14.7±2.7	b	18.8±1.7	b
Ca	–	19.9±3.5	a	22.7±8.0	a	27.4±5.6	a	18.7±5.8	a
	+	24.5±11.2	a	9.65±2.66	a	17.1±1.2	a	23.4±8.4	a
Mg	–	19.0±0.8	a	17.6±1.3	a	16.3±0.9	a	17.1±1.1	a
	+	18.6±0.9	a	15.5±0.7	b	15.9±0.5	b	16.8±0.5	ab
K	–	112±4	ab	102±5	a	113±3	ab	117±4	b
	+	112±5	a	96.7±6.5	ab	97.3±7.1	ab	88.8±4.9	b
Ca/Mn	–	13.7±2.1	a	1.69±0.46	b	1.83±0.25	b	1.71±0.46	b
	+	10.5±2.3	a	0.59±0.16	b	1.74±0.40	b	1.17±0.38	b
Mg/Mn	–	13.3±1.6	a	1.46±0.17	b	1.33±0.11	b	1.66±0.04	b
	+	9.18±1.85	a	1.00±0.08	b	1.44±0.52	b	0.93±0.10	b

Arithmetic mean ± S.E. Statistics: Duncan's multiple range test, $P \leq 0.05$, df = 16. Within a row, means sharing a common letter do not differ significantly.

[a] Recovery: +, 24 h recovery after treatment; –, without recovery day.

Table 9-2. Effects of species, duration and recovery day on intracellular and extracellularly bound content of Mn, Ca, Mg, K and on ratios of Ca and Mg to Mn in *Lobaria pulmonaria* and *Nephroma helveticum*

	Total		Species		Time		Recovery		Sp.×Time×Rec.	
	Var.[a]	*F*[b]	Var.	*F*	Var.	*F*	Var.	*F*	Var.	*F*
Intracellular:										
Mn	87	24.7***	44	336***	23	29.7***	13	97.1***	18	2.86***
Ca	18	0.91	1	0.93	4	0.89	1	1.56	27	0.88
Mg	25	1.40	0	0.07	8	2.00	1	1.35	7	1.28
K	46	3.51***	28	58.6***	2	0.83	4	7.37**	12	3.51***
Ca/Mn	46	3.03***	6	10.3**	23	6.71***	0	0.14	16	1.64
Mg/Mn	61	5.67***	6	19.4***	27	11.2***	0	0.00	11	3.50***
Extracellular:										
Mn	92	48.4***	1	11.4***	82	194***	6	83.1***	3	2.37**
Ca	91	41.3***	0	1.37	83	170***	6	68.6***	2	1.38
Mg	97	147***	3	110***	87	589***	4	152***	0	9.15***
Ca/Mn	84	20.3***	2	11.5***	53	58.4***	1	4.54*	28	9.56***
Mg/Mn	80	16.6***	1	7.01**	62	58.1***	0	2.74	16	4.68***

[a] Variance in percent.
[b] *F* value; levels of significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (ANOVA; df = 27; 1, 6, 19).

Table 9-3. Concentrations of extracellularly bound Mn, Ca, Mg, K (in μmol g^{-1} d. wt.) and molar ratios of Ca and Mg to Mn in *Lobaria pulmonaria*, *Nephroma helveticum* and *Leptogium saturninum* after treatment with 10 mM $MnCl_2$ alone or in combination with 2.5 mM $CaCl_2$ or 2.5 mM $MgCl_2$ for 40 min

	Rec.[a]	Control		Mn		Mn + Ca		Mn + Mg	
Lobaria pulmonaria:									
Mn	–	0.93±0.32	a	77.4±2.8	b	31.4±2.1	c	40.7±1.4	d
	+	0.31±0.04	a	60.2±2.9	b	26.5±0.5	c	33.7±1.3	d
Ca	–	31.1±2.3	a	11.4±0.9	b	12.8±4.5	b	16.9±7.7	b
	+	32.9±2.2	a	4.87±0.38	b	27.5±0.5	c	6.65±0.45	d
Mg	–	16.0±1.0	a	2.25±0.16	b	0.54±0.08	b	19.0±0.7	c
	+	15.3±0.7	a	5.43±0.17	b	2.30±0.07	c	23.8±0.8	d
Ca/Mn	–	47.3±11.1	a	0.06±0.00	b	0.43±0.16	b	0.42±0.20	b
	+	129±23	a	0.19±0.01	b	1.04±0.01	b	0.20±0.02	b
Mg/Mn	–	24.3±5.9	a	0.03±0.00	b	0.02±0.00	b	0.47±0.00	b
	+	54.9±9.1	a	0.09±0.00	b	0.09±0.00	b	0.71±0.01	b
Nephroma helveticum:									
Mn	–	1.10±0.21	a	74.1±10.8	b	56.3±1.6	c	56.4±1.8	c
	+	2.05±0.51	a	57.0±2.8	b	51.7±2.6	bc	48.5±1.3	c
Ca	–	28.5±3.1	a	11.3±2.6	b	14.8±1.1	b	4.34±0.76	c
	+	34.8±2.4	a	12.0±0.9	b	18.7±0.7	c	6.48±0.39	d
Mg	–	19.0±0.4	a	2.69±0.58	b	1.84±0.28	b	8.65±0.27	c
	+	24.2±1.6	a	5.24±0.26	b	3.24±0.13	b	9.27±0.55	c
Ca/Mn	–	36.3±14.1	a	0.17±0.05	b	0.26±0.01	b	0.08±0.01	b
	+	24.6±7.6	a	0.21±0.01	b	0.36±0.01	b	0.13±0.01	b
Mg/Mn	–	24.2±9.4	a	0.04±0.00	b	0.03±0.00	b	0.15±0.00	b
	+	16.0±6.8	a	0.09±0.00	b	0.06±0.00	b	0.19±0.01	b
Leptogium saturninum:									
Mn	–	0.95±0.29	a	162±7	b	146±4	b	159±7	b
	+	1.33±0.26	a	173±6	b	144±7	c	146±4	c
Ca	–	152±6	a	37.3±2.3	b	70.7±7.1	c	32.9±1.3	b
	+	140±8	a	43.5±3.7	b	72.5±4.3	c	44.0±3.2	b
Mg	–	69.7±2.2	a	12.7±1.1	b	11.0±1.7	b	33.5±6.6	c
	+	70.0±2.4	a	17.2±0.7	b	15.0±1.9	b	39.7±1.7	c
Ca/Mn	–	248±79	a	0.23±0.02	b	0.48±0.04	b	0.21±0.01	b
	+	158±70	a	0.25±0.01	b	0.51±0.03	b	0.30±0.03	b
Mg/Mn	–	111±34	a	0.08±0.01	b	0.07±0.01	b	0.20±0.04	b
	+	73.1±26.7	a	0.10±0.01	b	0.10±0.01	b	0.27±0.00	b

Arithmetic mean ± S.E. Statistics: Duncan's multiple range test, $P \leq 0.05$, df = 16. Within a row, means sharing a common letter do not differ significantly.

[a] Recovery: +, 24 h recovery after treatment; –, without recovery day.

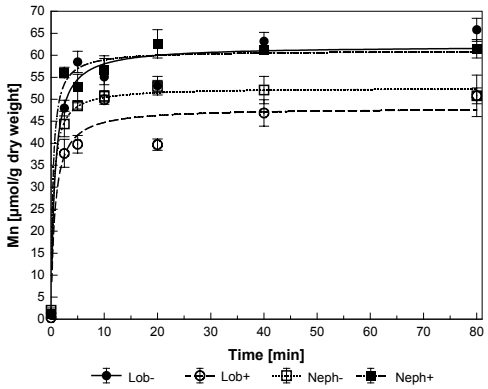

Fig. 9-2. Extracellular Mn adsorption in *Lobaria pulmonaria* and *Nephroma helveticum* related to time from 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob –, Neph –) or after a subsequent recovery day in the growth chamber (Lob +, Neph +). Arithmetic means of five replicates ± standard error.

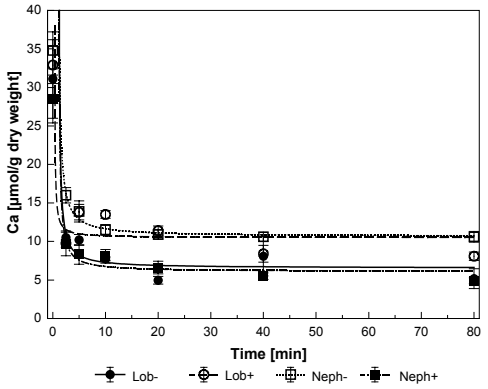

Fig. 9-3. Extracellularly bound Ca concentration in *Lobaria pulmonaria* and *Nephroma helveticum* related to time after incubation with 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob –, Neph –) or after a subsequent recovery day in the growth chamber (Lob +, Neph +). Arithmetic means of five replicates ± standard error.

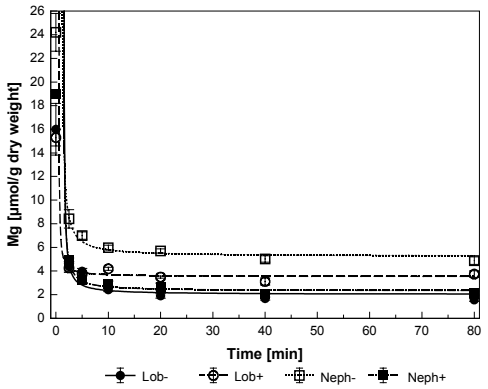

Fig. 9-4. Extracellularly bound Mg concentration in *Lobaria pulmonaria* and *Nephroma helveticum* related to time after incubation with 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob –, Neph –) or after a subsequent recovery day in the growth chamber (Lob +, Neph +). Arithmetic means of five replicates ± standard error.

3.2. Chlorophyll fluorescence

The effect of Mn incubation on the effective quantum yield of PS2 (Φ_2) increased in the order *L. pulmonaria* < *N. helveticum* < *L. saturninum*. In *L. pulmonaria*, only the samples exposed to 10 mM $MnCl_2$ exhibited a significantly reduced Φ_2 two weeks after incubation (Fig. 9-5). In *N. helveticum*, 10 mM $MnCl_2$ reduced Φ_2 already after two days significantly (Fig. 9-6). In *L. saturninum*, 10 mM $MnCl_2$ reduced Φ_2 after two days, as did 5 mM $MnCl_2$ after two weeks (Fig. 9-7). Φ_2 of the samples where $MnCl_2$ was applied in combination with Ca and/or Mg did not significantly differ from Φ_2 of the controls.

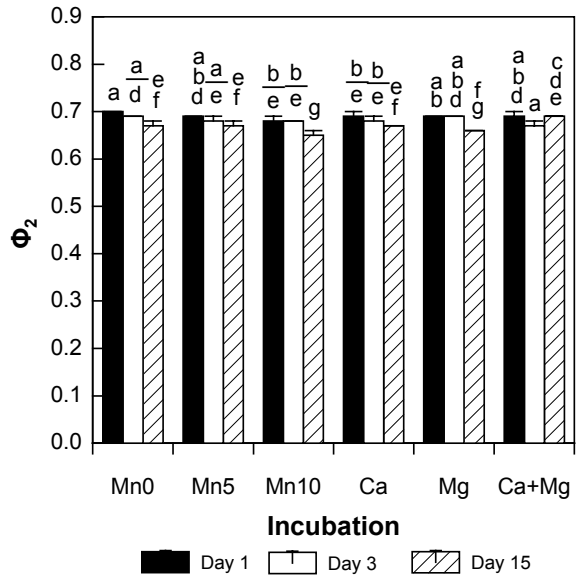

Fig. 9-5. Effective quantum yield of photochemical energy conversion in photosystem 2 (Φ_2) in *Lobaria pulmonaria* before (day 1) and two days (day 3) or two weeks (day 15) subsequent to 40 min incubation with either deionized water (Mn0), 5 or 10 mM $MnCl_2$ (Mn5, Mn10), 5 mM $MnCl_2$ and 2.5 mM $CaCl_2$ (Ca), 5 mM $MnCl_2$ and 2.5 mM $MgCl_2$ (Mg), or 5 mM $MnCl_2$, 1.25 mM $CaCl_2$ and 1.25 mM $MgCl_2$ (Ca+Mg). Significant differences are indicated by different letters above the colums (Duncan's multiple range test, $P \leq 0.05$, df = 160). A line between two letters above a column includes all letters in the alphabet between these letters. Vertical bars indicate ± standard error.

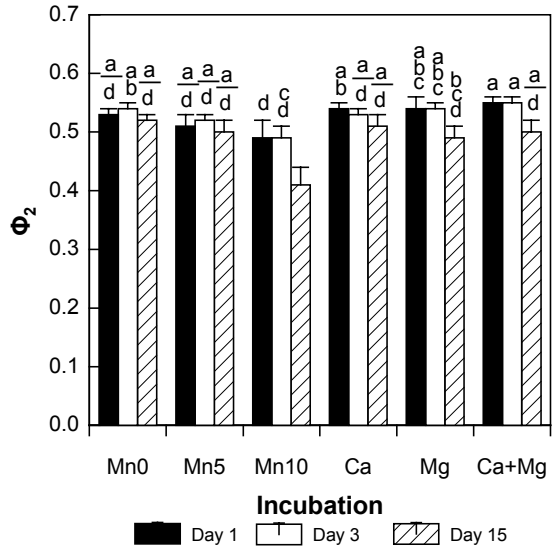

Fig. 9-6. Effective quantum yield of photochemical energy conversion in photosystem 2 (Φ_2) in *Nephroma helveticum* before (day 1) and two days (day 3) or two weeks (day 15) subsequent to 40 min incubation with either deionized water (Mn0), 5 or 10 mM $MnCl_2$ (Mn5, Mn10), 5 mM $MnCl_2$ and 2.5 mM $CaCl_2$ (Ca), 5 mM $MnCl_2$ and 2.5 mM $MgCl_2$ (Mg), or 5 mM $MnCl_2$, 1.25 mM $CaCl_2$ and 1.25 mM $MgCl_2$ (Ca+Mg). Significant differences are indicated by different letters above the colums (Duncan's multiple range test, $P \leq 0.05$, df = 160). A line between two letters above a column includes all letters in the alphabet between these letters. Vertical bars indicate ± standard error.

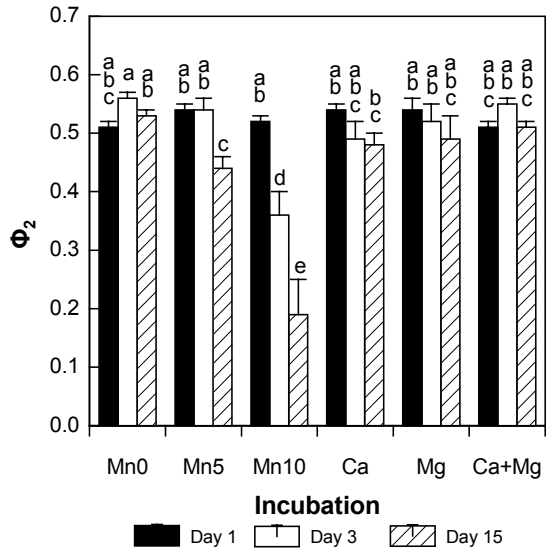

Fig. 9-7. Effective quantum yield of photochemical energy conversion in photosystem 2 (Φ_2) in *Leptogium saturninum* before (day 1) and two days (day 3) or two weeks (day 15) subsequent to 40 min incubation with either deionized water (Mn0), 5 or 10 mM $MnCl_2$ (Mn5, Mn10), 5 mM $MnCl_2$ and 2.5 mM $CaCl_2$ (Ca), 5 mM $MnCl_2$ and 2.5 mM $MgCl_2$ (Mg), or 5 mM $MnCl_2$, 1.25 mM $CaCl_2$ and 1.25 mM $MgCl_2$ (Ca+Mg). Significant differences are indicated by different letters above the colums (Duncan's multiple range test, $P \leq 0.05$, df = 160). Vertical bars indicate ± standard error.

4. Discussion

Incubation with $MnCl_2$ affected the quantum yield of PS2 increasingly in the order *L. pulmonaria* < *N. helveticum* < *L. saturninum*. This increase in sensitivity of the PS2 was correlated with increasing Mn uptake in the same order. While the heteromerous species *L. pulmonaria* and *N. helveticum* adsorbed very similar amounts of Mn extracellularly, intracellular Mn uptake in *N. helveticum* was twice as much as in *L. pulmonaria*. Both species transferred similar rates of extracellular Mn into the cell interior during the recovery day; thus, the species-specific difference in the intracellular Mn concentration was maintained. The stronger reduction of Φ_2 in *N. helveticum* than in *L. pulmonaria* could be due to the higher intracellular uptake of Mn. PAUL et al. (2003) attributed the high tolerance of the crustose green-algal lichen *Lecanora conizaeoides* to its ability to maintain its intracellular Mn concentrations low as a consequence of effective immobilization. Whether the higher intracellular Mn uptake in *N. helveticum* than in *L. pulmonaria* was due to different uptake rates of the mycobionts or to different uptake characteristics of the cyanobacterial photobiont in *N. helveticum* and the green-algal (quantitatively most important) primary photobiont in *L. pulmonaria* should be studied by X-ray microanalysis. When BROWN & BECKETT (1983) compared Zn concentrations of field-grown samples of several bipartite cyanolichens (including *N. laevigatum*), tripartite lichens (including *L. pulmonaria*) and bipartite chlorolichens, they found the tendency that bipartite chlorolichens contained less Zn than lichen species that contained cyanobacteria as the primary photobiont or in cephalodia. Further, incubation with $ZnSO_4$ reduced photosynthetic ^{14}C fixation more in cyanolichens than in chlorolichens (BROWN & BECKETT 1983).

The gelatinous, homoiomerous *L. saturninum* took up much more Mn both intra- and extra-cellularly than the heteromerous *L. pulmonaria* and *N. helveticum*. It is reasonable to assume that extracellular Mn adsorption in *L. saturninum* was not confined to cation exchange sites of the cell walls, but also took place in the exopolysaccharide matrix formed by the *Nostoc* cyanobiont, as PAUL et al. (2003) found significant adsorption of Mn to the cortical exopolysaccharide matrix in *Hypogymnia physodes*. The high uptake rate for Mn could be the cause for the relatively strong reduction of Φ_2 in Mn-exposed samples of *L. saturninum*. The results parallel results of BROWN & BECKETT (1983),

who found the gelatinous cyanolichens *Collema tenax* and *Lichina pygmaea* to be among those lichen species where $ZnSO_4$ reduced photosynthetic ^{14}C fixation most.

Based on our chlorophyll fluorescence data, Mn sensitivity increases in the expected order *L. pulmonaria* < *N. helveticum* < *L. saturninum*. So, these results suggest that the limitation of *Leptogium cellulosum*, *L. saturninum*, *Lobaria hallii*, *Nephroma helveticum*, *N. parile*, and *N. resupinatum* to conifer bark with the lowest Mn concentrations in the study of HAUCK & SPRIBILLE (2002) from northwestern Montana was causal. This supports the hypothesis of HAUCK & SPRIBILLE (2002) that the 'dripzone effect' of GOWARD & ARSENAULT (2000), i.e., the abundant occurrence of cyanolichens on conifers growing in the direct neighborhood of *Populus* in the Pacific Northwest of America, may be due to lower Mn concentrations of conifer bark within versus outside the dripzone of *Populus*. Such lower concentrations were found both by GOWARD & ARSENAULT (2000) and HAUCK & SPRIBILLE (2002). The lower sensitivity of Φ_2 in the tripartite lichen *L. pulmonaria* compared to *N. helveticum* and *L. saturninum* matches with the observation of HAUCK & SPRIBILLE (2002) that *L. pulmonaria* dwelled substrates with higher Mn concentration than the bipartite cyanolichens, while bark with the highest Mn content was on inhabited by bipartite chlorolichens. Combined results from the field and from the present experiments suggest that Mn sensitivity may increase from tripartite lichens via bipartite, heteromerous lichens towards bipartite, homoiomerous lichens. However, additional species should be studied to test whether this rule is generally valid. Φ_2 is, of course, only a single vitality parameter, but a very significant one (GARTY et al. 2000, 2002), which was selected, because it is non-invasive.

The alleviating effect $CaCl_2$ and $MgCl_2$ exerted on $MnCl_2$-induced reduction of Φ_2 agrees with results from *Hypogymnia physodes*, where $CaCl_2$ and $MgCl_2$ compensated for $MnCl_2$-induced chlorophyll degradation (HAUCK et al. 2002c, d). Further, the Mn/Ca ratio is known to be significant for photosynthesis, as both metals are involved in photosynthetic water oxidation. There is an optimum relationship between the ratio of Mn to Ca and photosynthetic oxygen evolution, because the ions compete for separate Mn^{2+} and Ca^{2+} binding sites (ONO & INOUE 1983, HOGANSON et al. 1989). Remarkably, neither $CaCl_2$ nor $MgCl_2$ reduced intracellular Mn uptake in *L. saturninum* or *N.*

helveticum, whereas there was significant reduction in *L. pulmonaria* and in *H. physodes* (HAUCK et al. 2002 d). Extracellular adsorption of Mn was reduced in all species by $CaCl_2$ and $MgCl_2$.

All species transferred apoplastic Mn into the cells during the recovery day subsequent to the exposure to $MnCl_2$ solution. This agrees with data of BROWN & BECKETT (1985), who observed that intracellular Cd concentration was still increasing after 96 h in *Cladonia portentosa* or 124 h in *Peltigera horizontalis*, after lichen pieces had been shaken with 50 µM $CdSO_4$ for only 10 min. These results are of high ecological significance, as they show that metal ions can still invade the cell a considerable while after the actual exposure, even though the original source may be long absent. Mn is often leached from the canopy in pulses, especially at temperatures around the freezing point in winter time (LEVIA & HERWITZ 2000). In stemflow of *Picea abies* forests in the Harz Mountains, Germany, peak concentrations of Mn were up to 12 times higher than mean concentration (HAUCK 2000). The present data in combination with those of BROWN & BECKETT (1985) suggest that such short peaks in Mn concentration, which last for minutes or hours, may affect lichen metabolism for extended periods.

Summarizing, our results suggest that the localization of cyanolichens on bark with low Mn concentration on coniferous trees in northwestern Montana may be due to their high Mn sensitivity, as assessed from chlorophyll fluorescence data. The tripartite *L. pulmonaria* apparently occupies an intermediate position between bipartite cyanolichens and chlorolichens in the field (HAUCK & SPRIBILLE 2002) and is less sensitive to Mn in terms of reduction of Φ_2 than the bipartite cyanolichens *L. saturninum* and *N. helveticum*. Translocation of Mn in moist thallus pieces of *L. pulmonaria*, *N. helveticum* and *L. saturninum* from the apoplast to the symplast during recovery after incubation with $MnCl_2$ suggests that short exposures to Mn in the field may affect lichen metabolism for long periods.

Chapter 10

Effects of manganese on chlorophyll fluorescence in epiphytic cyano- and chloro-lichens

Abstract

Incubation with 10 mM $MnCl_2$ for 1 h decreased the effective quantum yield of photochemical energy conversion in photosystem 2 in the bipartite chlorolichen *Hypogymnia physodes* as well as in the bipartite cyanolichens *Leptogium saturninum* and *Nephroma helveticum,* but not in the tripartite lichen *Lobaria pulmonaria.* Among the bipartite species, Mn sensitivity increased in the order *H. physodes* < *N. helveticum* < *L. saturninum.* This equals the sequence heteromerous chlorolichen < heteromerous cyanolichen < homoiomerous (gelatinous) cyanolichen. $MnCl_2$ reduced non-photochemical quenching of chlorophyll fluorescence in the bipartite cyanolichens and in *H. physodes;* in the latter, however, this decrease was limited to light intensities above the adapted growth light intensity. Photochemical quenching was increased in *H. physodes,* but reduced in the bipartite cyanolichens. The results indicate that the bipartite cyanolichens *L. saturninum* and *N. helveticum* are even more sensitive to high Mn concentrations than the chlorolichen *H. physodes,* the low Mn tolerance of which has been already demonstrated. This agrees with results of field studies from western North America, where conifer bark under cyanolichens (including *L. saturninum* and *N. helveticum)* was found to contain less Mn than bark which only supported chlorolichens. The high sensitivity of the bipartite cyanolichens can be attributed to a high sensitivity of the *Nostoc* photobiont, as sensitivity was highest in the gelatinous *L. saturninum,* which has a higher photobiont/mycobiont ratio than the less sensitive homoiomerous *N. helveticum.* The high Mn tolerance of *L. pulmonaria* is probably not due to its being a tripartite lichens, but might be caused by high tolerance of the green-algal primary photobiont *Dictyochloropsis,* which is, however, not experimentally

proven. The high Mn tolerance of the highly SO_2-sensitive *L. pulmonaria* shows that different mechanisms are responsible for Mn and SO_2 toxicity in lichens.

1. Introduction

Combination of field studies and experimental work showed that epiphytic chlorolichen abundance in coniferous forests of Europe and North America is, among other factors, controlled by the supply with Mn (HAUCK 2003). Cover of many lichen species decreased with increasing Mn concentration in bark (HAUCK et al. 2001b, SCHMULL & HAUCK 2003a) or stemflow (HAUCK et al. 2002a, SCHMULL et al. 2002). *Hypogymnia physodes* was selected as a model for an apparently Mn-sensitive foliose, epiphytic chlorolichen species for experimental work. Damage symptoms found in this species at high Mn concentrations were, e.g., chlorophyll and chloroplast degradation as well as inhibition of soredia growth and of the formation of autospores in the *Trebouxia* photobiont (HAUCK et al. 2002c, d, 2003). Further, high Mn concentrations apparently interrupt the water and assimilate flux between the bionts, as the physical contact between them is reduced and the mesosome-like structures, which are thought to be involved in metabolite transfer and storage, are degraded (PAUL et al. 2004). The decrease of concentric bodies in Mn-exposed soredia of *H. physodes* suggests that their ability to withstand the repeated drying and re-wetting cycles is reduced (PAUL et al. 2004). It is obvious from these results that *H. physodes* is a chlorolichen, the abundance of which is controlled by high Mn concentrations in the field. These high Mn concentrations occur naturally, as the Mn in the bark derives from soil-borne Mn taken up by the tree root (LÖVESTAM et al. 1990, SLOOF & WOLTERBEEK 1993, HAUCK et al. 2002a). Moreover, important proportions of the Mn in stemflow originates from soil-borne Mn that is leached from the tree (LEVIA & HERWITZ 2000). While several chlorolichen species are thought to behave similarly in terms of Mn sensitivity like *H. physodes*, the crustose, epiphytic chlorolichen *Lecanora conizaeoides* was found to be highly Mn-tolerant both in the field (HAUCK et al. 2001b) and in the experiment (HAUCK et al. 2003), probably primarily due to effective immobilization of Mn within the fungal hyphae and in the apothecia in particular (PAUL et al. 2003).

While, so far, studies to Mn toxicity in epiphytic lichens primarily referred to chlorolichens, field data from coniferous forests of western North America suggested that epiphytic cyanolichens could be particularly sensitive to high Mn supply (HAUCK & SPRIBILLE 2002). Therefore, HAUCK et al. (2005) studied Mn uptake from $MnCl_2$ solution in epiphytic cyanolichens. In the present study, we investigated Mn toxicity in three different types of cyanolichen species that aroused suspicion of being Mn-sensitive in the field study of HAUCK & SPRIBILLE (2002). These species were (1) *Leptogium saturninum* as an example for a bipartite, homoiomerous cyanolichen with a gelatinous thallus, (2) the bipartite, heteromerous, non-gelatinous *Nephroma helveticum*, and (3) the tripartite, heteromerous, non-gelatinous *Lobaria pulmonaria*, which contains a green alga as the primary photobiont and a cyanobacterium in internal cephalodia. *H. physodes* was studied in comparison as a heteromerous, chlorolichen species already known to be Mn-sensitive.

Since degradation of the concentrations of Chl *a* and *b* and of the chloroplasts due to high Mn concentrations is pronounced in *H. physodes* (HAUCK et al. 2002c, 2003, PAUL et al. 2004), we selected chlorophyll fluorescence as method to compare Mn sensitivity in our study species. Chlorophyll fluorescence is well proven to quantify the effect of transition metal toxicity in lichens (GARTY 2001). The objective of the study was to test the hypothesis that chlorophyll fluorescence in the studied lichens species is increasingly sensitive to high Mn supply in the order heteromerous chlorolichen (*H. physodes*) < tripartite, heteromerous cyanolichen (*L. pulmonaria*) < bipartite, heteromerous cyanolichen (*N. helveticum*) < bipartite, homoiomerous cyanolichen (*L. saturninum*). This hypothesis is based on the increasing dominance of cyanobacteria in the lichens from *H. physodes* (absent) via *L. pulmonaria* (few in cephalodia) to *N. helveticum* (cyanobacteria concentrated on photobiont layer) and *L. saturninum* (cyanobacteria ubiquitous in the thallus).

2. Materials and Methods

Abbreviations: CCM = CO_2-concentrating mechanism; Chl = chlorophyll; Fo = minimum Chl fluorescence yield in the dark-adapted state; Fo' = minimum Chl fluorescence yield in the light-adapted state; F_M = absolute maximum Chl fluorescence yield; F_M' = maximum Chl fluorescence yield in the light-adapted state; $F_{M\ dark}'$ = maximum Chl fluorescence yield in the dark-adapted state (= F_M in chlorolichens; < F_M in cyanolichens); Fs = steady-state Chl fluorescence yield in the light-adapted state; Fv = maximum variable Chl fluorescence yield in the dark-adapted state; NPQ = non-photochemical Chl fluorescence quenching; PPFD = photosynthetic photon flux density; PS1 = photosystem 1; PS2 = photosystem 2; qN = non-photochemical quenching of variable Chl fluorescence; qP = photochemical quenching of variable Chl fiuorescence; Φ_2 = effective quantum yield of photochemical energy conversion in PS2.

2.1. Lichen sampling and incubation procedure

Thalli of *Leptogium saturninum* (Dickson) Nyl., *Lobaria pulmonaria* (L.) Hoffm. and *Nephroma helveticum* Ach. were sampled from conifer bark in British Columbia, Canada. Samples of *L. saturninum* and *N. helveticum* were taken in Wells-Gray Provincial Park, ca. 25 km N Clearwater, 51°49' N, 120°07' W, 820 m, those of *L. pulmonaria* in Spahats Provincial Park, N Clearwater, 51°45' N, 120°00' W, 760 m. *Hypogymnia physodes* (L.) Nyl. was collected in the Harz Mountains, Germany (Rotes Bruch near Benneckenstein, 52°4' N, 10°43' E). The primary photobiont of *H. physodes* is *Trebouxia* (AHMADJIAN 1993), that of *L. pulmonaria Dictyochloropsis* (SCHOFIELD et al. 2003) and that of *L. saturninum* and *N. helveticum* is *Nostoc* (RIKKINEN 2002). The latter is also the secondary photobiont of *L. pulmonaria*. Air-dry thalli were stored in the dark at room temperature for a few days subsequent to collection (and during transport by aircraft in the case of the cyanolichens) and were then frozen at -30 °C.

Thallus pieces of up to 5 cm^2 with a vital appearance were preincubated on moist filter paper in Petri dishes at 80 % relative humidity, a day temperature (for 13 hours daily) of 13 °C during a photon flux of 15 µmol m^{-2} s^{-1}, and a night temperature of 10 °C for 1 week. Recovery of chlorophyll fluorescence after freezing during the preincubation

period was controlled by repeated measurements. After preincubation, thallus pieces were exposed to 25 ml of 10 mM $MnCl_2$ or deionized water for 1 h. All solutions were adjusted to pH 5 with NaOH and HCl. Subsequent to the exposure to metal solution or water, samples were briefly washed in deionized water and then placed on moist filter paper again at climatic conditions as described above. The experiment was carried out in triplicate.

2.2. Measurement and calculation of chlorophyll fluorescence parameters

Chlorophyll fluorescence was measured with a pulse amplitude modulated fluorimeter (Mini-PAM Photosynthesis Yield Analyzer, Walz Mess- und Regeltechnik, Effeltrich, Germany) on the days following the $MnC1_2$ exposure. The measuring beam had a light intensity of c. 0.14 µmol m^{-2} s^{-1} from a light-emitting dioide (peak emission 650 nm). Measuring frequency was 0.6 kHz at dark. In actinic light, the measuring beam was modulated at 0.6 kHz in chlorolichens, but at 20 kHz cyanolichens to improve the signal/noise ratio. Saturation light was applied as a pulse of 6000 µmol m^{-2} s^{-1} for 400 ms. This intensity was tested to saturate fluorescence emission without causing photoinhibition (SCHREIBER et al. 1995). Nomenclature of chlorophyll fluorescence parameters follows ROHÁCEK (2002). Dark adaptation of chlorolichens before measurements lasted for 30 min (MACKENZIE et al. 2001), whereas 15 min were sufficient for cyanolichens (CAMPBELL et al. 1998). The first measurement was carried out at dark for determining Fo. Subsequent to a saturation pulse followed the determination of $F_{M\ dark}$', which equals F_M in chlorolichens, but not in cyanolichens (CAMPBELL et al. 1998). In the latter, several consecutive $F_{M\ dark}$' values were measured at low PPFD. The highest of these values was then used for calculations instead of true F_M as proposed by SUNDBERG et al. (1997). Fs was measured in actinic light by stepwise increasing PPFD and allowing the lichens to adapt to the respective light intensity for 150 s in cyanolichens and 210 s in chlorolichens. F_M' was determined following a saturation pulse. Fo' was calculated according to OXBOROUGH & BAKER (1997) with the equation $Fo' = Fo/([Fv/F_M] + [Fo/F_M'])$. Fv is defined as $F_M - Fo$ (ROHÁCEK 2002); F_M had to be replaced by $F_{M\ dark}$' in cyanolichens (CAMPBELL et al. 1998). In the case of *H. physodes, L. pulmonaria* and *N. helveticum,* a close correlation was found between calculated Fo' and values measured after keeping light-adapted samples for 5 s at dark (*r*

= 0.85 – 0.96; $P < 0.001$), whereas correlation was not satisfactory in the case of *L. saturninum* ($r = 0.68$; $P < 0.001$). Calculated values were preferred, as the measurement after 5 s can only be started manually with the Mini-PAM and is, thus, a potential source of error. The above fluorescence parameters were used to calculate Φ_2, qP, qN, and NPQ according to the following equations (GENTY et al. 1989, BILGER & BJÖRKMAN 1990, VAN KOOTEN & SNEL 1990): $\Phi_2 = (F_M' - Fs)/F_M'$; $qP = (F_M' - Fs)/(F_M' - Fo')$; $qN = 1- (F_M' - Fo')/(F_M - Fo)$; $NPQ = (F_M - F_M')/ F_M'$.

2.3. Statistics

The effect of incubation with $MnCl_2$ on fluorescence parameters was quantified with an analysis of variance (ANOVA). Type III sums of squares were used for calculating *F* values. Aside from the $MnCl_2$ concentration of the incubation medium, PPDF and interaction between $MnCl_2$ concentration and PPFD were included in the analysis, but are not shown in Table 10-1. The ANOVA was calculated with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.).

3. Results

Φ_2 in *H. physodes* and *L. pulmonaria,* which contain a green-algal primary photobiont, steadily decreased with increasing PPFD (Fig. 10-1).

In *N. helveticum* and *L. saturninum,* which have a cyanobacterial primary photobiont, Φ_2 increased at low light intensities until a PPFD near the acclimated growth light intensity of 15 µmol m^{-2} s^{-1} was reached. Above growth light intensity, Φ_2 decreased as in the chlorolichens and tripartite lichens.

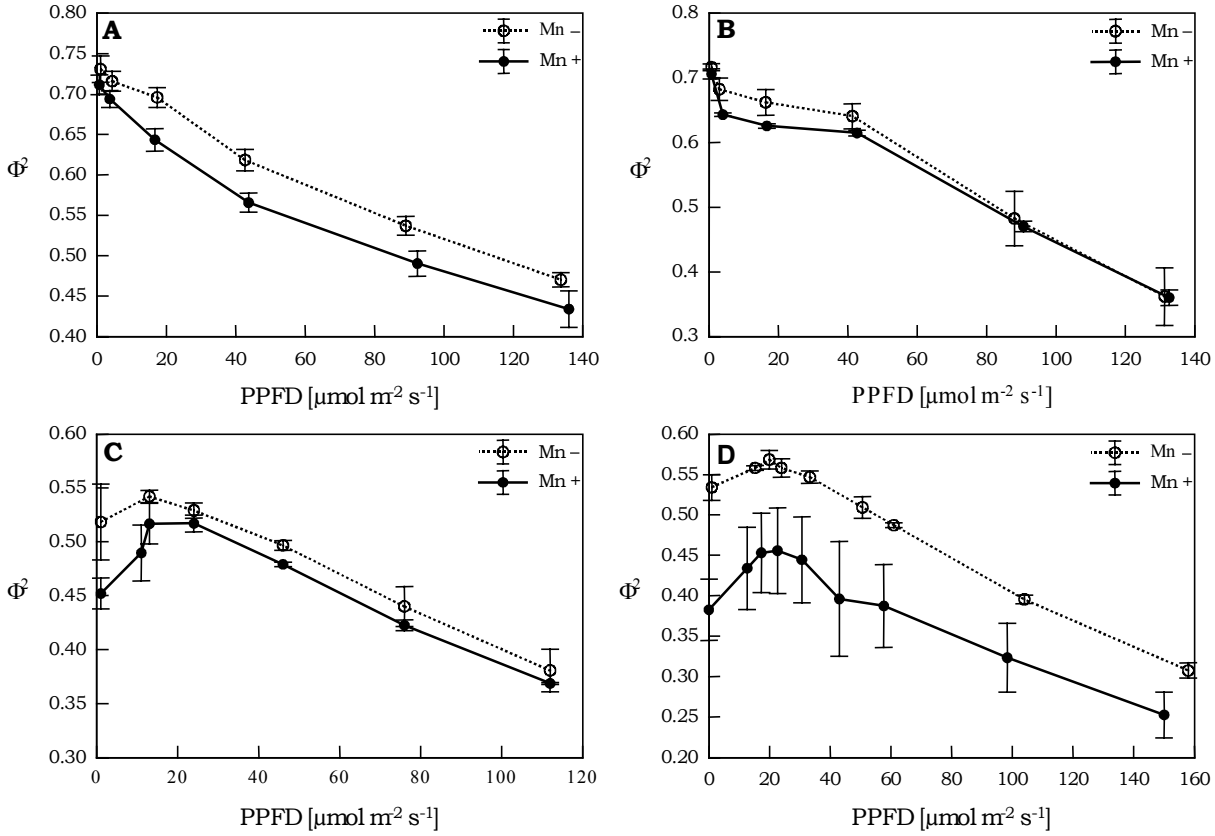

Fig. 10-1. Effective quantum yield of photochemical energy conversion in PS2 (Φ_2) in samples of the heteromerous, bipartite chlorolichen *Hypogymnia physodes* (A), the heteromerous, tripartite lichen *Lobaria pulmonaria* (B), the heteromerous, bipartite cyanolichen *Nephroma helveticum* (C) and the homoiomerous, bipartite cyanolichen *Leptogium saturninum* (D) after incubation with 10 mM $MnCl_2$ (Mn +) or deionized water (Mn –) for 1 h. Error bars indicate standard errors of three replicate samples.

$MnC1_2$ significantly reduced Φ_2 in *H. physodes, N. helveticum* and *L. saturninum,* but not in *L. pulmonaria* (Fig. 10-1, Table 10-1); the decline in *L. saturninum* was most significant. A strong influence of $MnCl_2$ on Φ_2 in *L. saturninum* was also observed for chlorophyll fluorescence induction kinetics (Fig. 10-2). Both in the light and in the dark, Φ_2 was reduced by about 50 %. For *H. physodes,* a less severe reduction was observed (Fig. 10-2).

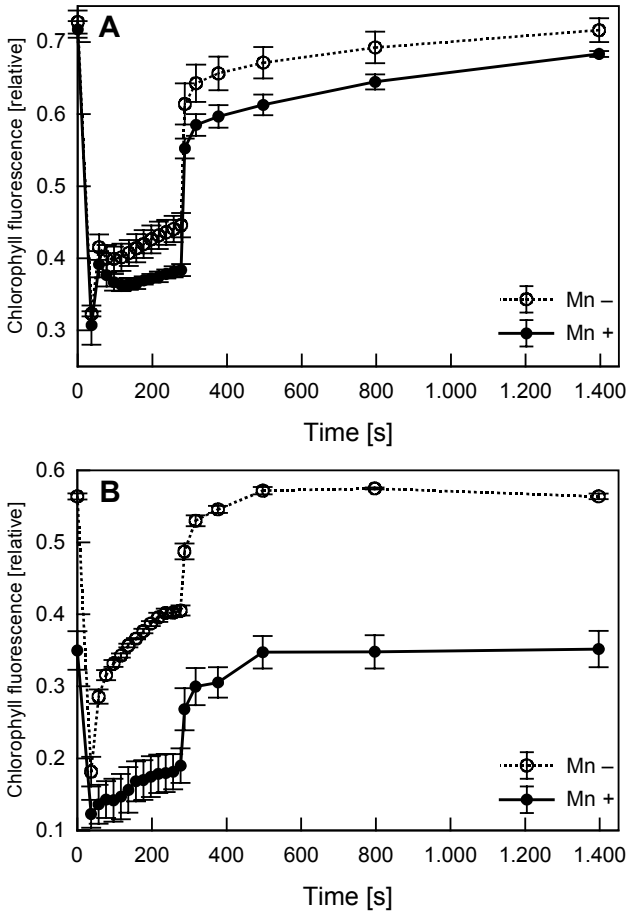

Fig. 10-2. Slow chlorophyll fluorescence induction kinetics in samples of the heteromerous, bipartite chlorolichen *Hypogymnia physodes* (A) and the homoiomerous, bipartite cyanolichen *Leptogium saturninum* (B) after incubation with 10 mM $MnCl_2$ (Mn +) or deionized water (Mn –) for 1 h. Error bars indicate standard errors of three replicate samples.

Like Φ_2, non-photochemical quenching was principally different between the lichens with green-algal and cyanobacterial primary photobiont. This concerned both qN (Fig. 10-3) and NPQ. In *H. physodes* and *L. pulmonaria,* the non-photochemical quenching continuously increased with increasing PPFD starting from zero. In *L. saturninum* and *N. helveticum,* the non-photochemical quenching achieved its maximum in dark-adapted

thalli and decreased to a minimum at low light intensities around 15 µmol m^{-2} s^{-1}. At higher PPFD, non-photochemical quenching increased to levels in the magnitude of or somewhat below the value of dark-adapted thalli. $MnCl_2$ had no effect on qN in *L. pulmonaria,* but increased qN significantly in *H. physodes* and *N. helveticum* (Fig. 10-3, Table 10-1). High variation was apparently the cause that a trend to elevated qN in $MnCl_2$ treated samples of *L. saturninum* was statistically insignificant (Fig 10-3, Table 10-1). NPQ values were only significantly increased in $MnCl_2$ exposed samples of *H. physodes,* but not of the other species (Table 10-1).

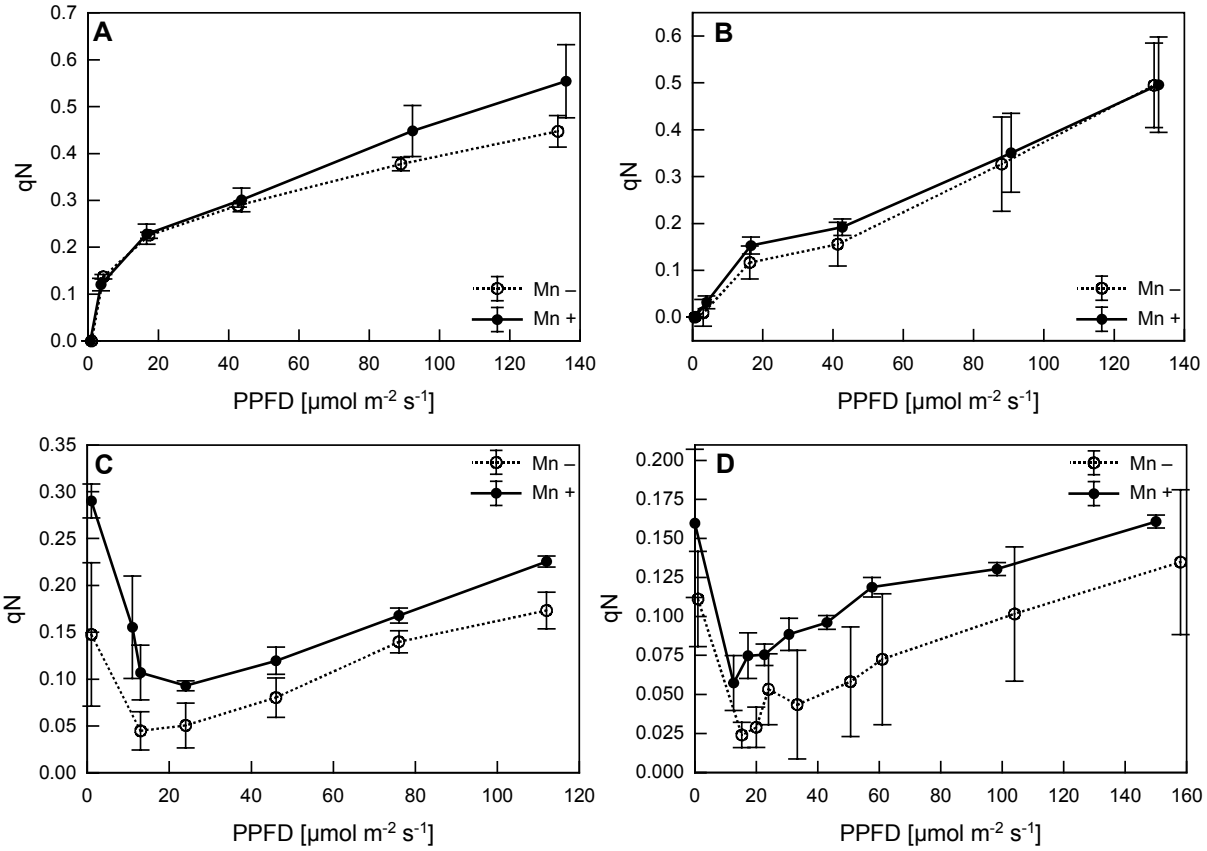

Fig. 10-3. Non-photochemical quenching of variable chlorophyll fluorescence (qN) in samples of the heteromerous, bipartite chlorolichen *Hypogymnia physodes* (A), the heteromerous, tripartite lichen *Lobaria pulmonaria* (B), the heteromerous, bipartite cyanolichen *Nephroma helveticum* (C) and the homoiomerous, bipartite cyanolichen *Leptogium saturninum* (D) after incubation with 10 mM $MnCl_2$ (Mn +) or deionized water (Mn –) for 1 h. Error bars indicate standard errors of three replicate samples.

The MnCl2-induced increase of qN and NPQ was effective only at light intensities exceeding 40 µmol m^{-2} s^{-1}.

The behavior of qP versus PPFD resembled that of Φ_2 in both the lichen species with green-algal and cyanobacterial photobiont (Fig. 10-4). The effect of $MnCl_2$ on qP differed between chloro- and cyano-lichens. qP of $MnCl_2$ treated samples was significantly lower than the control in *H. physodes* (except for high light intensities), equaled the control in *L. pulmonaria,* but was significantly (*N. helveticum)* and insignificantly (L. *saturninum)* higher than the control in the bipartite cyanolichens (Table 10-1).

Table 10-1. ANOVA results for the statistical significance of the effect of the incubation with Mn on selected chlorophyll fluorescence parameters.

Fluorescence parameter	Species	*F* value	*P*
Φ_2	*H. physodes*	12.2	*
	L. pulmonaria	0.00	
	N. helveticum	8.33	**
	L. saturninum	16.3	***
NPQ	*H. physodes*	599	***
	L. pulmonaria	0.00	
	N. helveticum	1.63	
	L. saturninum	0.39	
qN	*H. physodes*	264	***
	L. pulmonaria	0.01	
	N. helveticum	14.4	***
	L. saturninum	0.04	
qP	*H. physodes*	126	***
	L. pulmonaria	0.67	
	N. helveticum	6.78	*
	L. saturninum	0.30	

Note: Significant differences from the control are indicated by * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$). *F* values are calculated from Type III sums of squares.

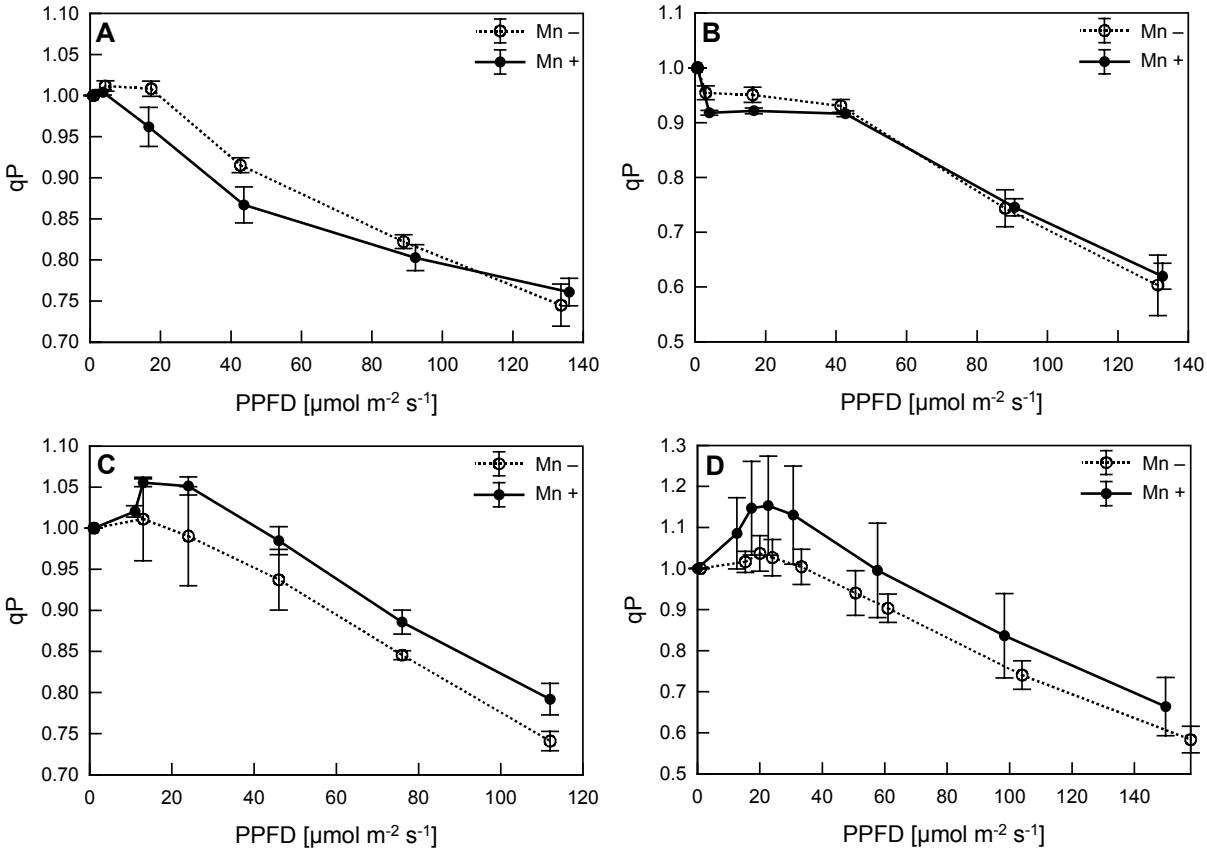

Fig. 10-4. Photochemical quenching of variable chlorophyll fluorescence (qP) in samples of the heteromerous, bipartite chlorolichen *Hypogymnia physodes* (A), the heteromerous, tripartite lichen *Lobaria pulmonaria* (B), the heteromerous, bipartite cyanolichen *Nephroma helveticum* (C) and the homoiomerous, bipartite cyanolichen *Leptogium saturninum* (D) after incubation with 10 mM $MnCl_2$ (Mn +) or deionized water (Mn –) for 1 h. Error bars indicate standard errors of three replicate samples.

4. Discussion

The characteristics of chlorophyll fluorescence parameters differ principally between chloro- and cyano-lichens or between green algae and cyanobacteria. This is because of the presence of phycobiliproteins, the use of a common electron transport chain both for photosynthesis and respiration, the availability of a CO_2-concentrating mechanism (CCM), which is more effective than in CCM-possessing green algae, and the ability to maintain PS2 centers open even under conditions of excess excitation (BADGER et al. 1993, SUNDBERG et al. 1997, CAMPBELL et al. 1998). While non-photochemical quenching in green algae and higher plants is primarily attributed to thermal dissipation (via the xanthophyll cycle; DEMMIG-ADAMS et al. 1990, HEBER et al. 2000), non-photo-

chemical quenching in cyanobacteria results from a state transition, which transfers excitation energy from the phycobilisome/PS2 supracomplex to PS1 (MILLER et al. 1991, SUNDBERG et al. 1997). Excitation energy is increasingly transferred to PS1 when the electron transport chain is reduced (CAMPBELL & ÖQUIST 1996). The necessity to equilibrate excitation energy between the photosystems in cyanobacteria derives from the fact that the redox state of the electron transport chain depends not only on photosynthesis, but also on respiration. Since respiration reduces the electron transport proteins at dark (MULLINEAUX & ALLEN 1986), non-photochemical quenching is highest at 0 μmol m^{-2} s^{-1}. With increasing oxidation of the electron transport chain in the light, the transfer of excitation energy to PS1 and, with it, the non-photochemical quenching is reduced (CAMPBELL & ÖQUIST 1996). These processes explain the decrease of qN in Fig. 10-3 and NPQ (not shown) in *L. saturninum* and *N. helveticum* from dark to the acclimated growth light intensity, while qN and NPQ steadily increased with increasing PPFD in the chlorolichen *H. physodes.* The constant increase of qN and NPQ in *L. pulmonaria* shows that the fluorescence signal in this tripartite lichen was only significantly influenced by its green-algal primary photobiont, but not by the cyanobacteria of the internal cephalodia. This result parallels results from *Nephroma arcticum,* which contains the green alga *Coccomyxa* as primary photobiont and the cyanobacterium *Nostoc* in internal cephalodia (SUNDBERG et al. 1997). In cephalodia, *Nostoc* is primarily specialized to nitrogen fixation (HYVÄRINEN et al. 2002).

The state transition in cyanobacteria is the reason why the quantum yield of PS2 noncyclic electron transport and the photochemical quenching increased from dark to growth light intensity in *L. saturninum* and *N. helveticum,* before these values started to decrease with increasing light intensity like in *H. physodes* and *L. pulmonaria* Photochemical quenching typically reacts quite differently on increasing PPFD in cyanobacteria than in green algae and higher plants. qP indicates the proportion of open PS2 reaction centers with the primary quinone-type electron acceptor in the oxidized state (ROHÁCEK 2002). Cyanobacteria have a high capacity to remove electrons from the PS2 with O_2 as the terminal electron acceptor (CAMPBELL et al. 1998). Thereby, they can keep the PS2 open even at PPFD values that are ten times higher than growth light intensity. This typically leads to constant qP values over this range of PPFD (BADGER & SCHREIBER 1993). Since green algae and higher plants lack this capability of discharging the PS2 and have, moreover, a lower PS1/PS2 ratio, qP in these organisms

falls steadily with increasing PPFD (CAMPBELL et al. 1998). SUNDBERG et al. (1997), however, showed that the light response of qP in lichens can considerably deviate from the ideal patterns of free-living algae, when they investigated cyanolichens of the genera *Leptogium, Lobaria, Nephroma* (but not the species used in the present study) and *Peltigera.* qP in *N. helveticum* showed a typical cyanolichen pattern, as found, e.g., in *Leptogium coralloideum* by SUNDBERG et al. (1997). The response of qP to PPFD in *L. saturninum,* however, was different, as qP decreased to 0.6 near 150 µmol m^{-2} s^{-1}. SUNDBERG et al. (1997) found a similar light response curve of qP in *Peltigera neopolydactyla.* These species mediate between the typical green-algal and cyanobacterial patterns. *H. physodes* did not show a typical green-algal response, as qP was lowered only to c. 0.75 at light intensities near 130 µmol m^{-2} s^{-1}.

The sensitivity of the chlorophyll fluorescence parameters to the incubation with $MnCl_2$ clearly differs between species. While *H. physodes, L. saturninum* and *N. helveticum* were differently sensitive to $MnCl_2$, none of the investigated parameters was changed by $MnCl_2$ in *L. pulmonaria.* This result is remarkable, as *L. pulmonaria* is very sensitive to SO_2 (TÜRK et al. 1974, WIRTH & TÜRK 1975) and is, thus, often taken as a classical example of an epiphytic lichen species of extremely low toxitolerance (WIRTH 1992, CONTI & CECCHETTI 2001). The present results show that this low toxitolerance is apparently not applicable to Mn. This implies that different mechanisms are responsible for Mn and SO_2 toxicity symptoms in lichens. This conclusion is supported by the high sensitivity of *H. physodes* to excess concentrations of Mn, which is evident from the present and from previous studies (HAUCK et al. 2002c, d, 2003, PAUL et al. 2003, 2004); *H. physodes* is considerably less sensitive to SO_2 than *L. pulmonaria* (TÜRK et al. 1974). An interesting question, which arises from the high Mn tolerance of *L. pulmonaria* is whether this species is generally insensitive to high concentrations of transition metals, which are known to be toxic to other lichen species, such as Cu, Pb and Ni. The mechanism causing the high tolerance to Mn in *L. pulmonaria* is unknown. Tolerance could either be due to effective immobilization of excess Mn in the mycobiont, as found for *L. conizaeoides* (PAUL et al. 2003), or to high tolerance of the primary photobiont *Dictyochloropsis.* Principal physiological differences are already known between *Dictyochloropsis* and *Trebouxia,* as the former, but not the latter lacks a CCM (PALMQVIST et al. 1998), so that differences in Mn tolerance between these green-

algal genera (and even more between *Dictyochloropsis* and *Nostoc)* are easily conceivable.

Among the studied lichen species, which were found to be Mn-sensitive, the impact of the $MnCl_2$ increases in the order *H. physodes* < *N. helveticum* < *L. saturninum*. This is, e.g., suggested by the increasing level of significance for the difference of Φ_2 between $MnCl_2$ treated and untreated samples in this order. The difference for *H. physodes* is significant on the 5 % level, that for *N. helveticum* on the I % level and that for *L. saturninum* on the 0.1 % level (Table 10-1). The reduced Φ_2 in $MnCl_2$ treated samples concomitant to increased qN shows that excess Mn reduces the photosynthetic capacity in these lichens in addition to the $MnCl_2$-induced reduction of chlorophyll concentrations and the degradation of thylakoids, which was found in *H. physodes* (HAUCK et al. 2002d, 2003; PAUL et al. 2004). The higher sensitivity of the homoiomerous *L. saturninum* than of the heteromerous *N. helveticum* suggests that the higher Mn sensitivity of these cyanolichen species than of the species with green-algal primary photobiont can be attributed to an especially high Mn sensitivity of *Nostoc,* as the ratio between *Nostoc* and fungal cells is higher in *L. saturninum* than in *N. helveticum*. Furthermore, the *Nostoc* cells can be supposed to be more sheltered from the environment in the heteromerous *N. helveticum* than in the homoiomerous *L. saturninum.*

Remarkably, $MnCl_2$ increased the non-photochemical quenching in *H. physodes* only at relative high light intensities, exceeding more than three times the acclimated growth light intensity. This suggests that *H. physodes* from shady sites could be more susceptible to rapid increases of PPFD at Mn-rich than at Mn-poor microsites. Correlation of high fluorescence yield with low qN and qP as found for the $MnCl_2$ supplemented and unsupplemented bipartite cyanolichens in the present study was already shown in a different context for different strains of the cyanobacterium *Synechococcus* (CAMPBELL et al. 1996).

5. Conclusions

The results support the initial hypothesis that cyanolichens are generally more sensitive to high Mn concentrations than chlorolichens. In this point, the experiment supports the hypothesis of HAUCK & SPRIBILLE (2002) that the limitation of epiphytic bipartite cyanolichens to conifer bark with low Mn content in the Pacific Northwest of North America can be mechanistically explained by low Mn tolerance of these lichens. However, the present results did not support the hypothesis that the tripartite, *Dictyochloropsis* and *Nostoc*-containing lichen *L. pulmonaria* occupies an intermediate position in terms of Mn sensitivity between bipartite chloro- and cyanolichens. Rather, the species turned out to be more Mn tolerant than the chlorolichen *H. physodes*. The low Mn sensitivity of the highly SO_2-sensitive *L. pulmonaria* as well as the high Mn sensitivity of the less SO_2-sensitive *H. physodes* give evidence of different mechanisms causing Mn and SO_2 related damage in lichens.

Chapter 11

Imaging effects of manganese on photosynthetic activity in epiphytic chloro- and cyano-lichens using fluorescence microscopy

Abstract

Chlorophyll fluorescence microscopy was applied to assess the sensitivity to excess concentrations of Mn in five species of epiphytic lichens that are characteristic of central European and North American montane conifer forests. The intensity of the fluorescence signal is a measure for the efficiency of photosystem 2 (PS2). Different sensitivity of the PS2 towards Mn was found for cyano- and chloro-lichens. The fluorescence signal in the gelatinous, homoiomerous *Leptogium saturninum* was most strongly affected by excess Mn. Reduced intensity of the fluorescence signal was also visually perceptible in Mn treated thalli of the heteromerous *Nephroma helveticum*, but high variability was apparently responsible for lacking statistical significance, which was calculated from means of brightness values of grayscale-converted fluorescence images. The heteromerous chlorolichen *Hypogymnia physodes* showed a significant reduction of the intensity of the fluorescence signal, though this was hardly visually perceptible on the fluorescence microscopy images. The fluorescence signal was as insensitive to excess Mn in the tripartite, heteromerous *Lobaria pulmonaria* as in the homoiomerous chlorolichen *Lecanora conizaeoides*. The Mn tolerance of *L. pulmonaria* can be due to either high Mn tolerance of the primary photobiont *Dictyochloropsis* or to effective intrathalline immobilization of Mn by the mycobiont. The latter is known to be the cause of high Mn tolerance in *L. conizaeoides*. The high Mn tolerance of *L. pulmonaria* shows that mechanisms leading to Mn sensitivity in lichens are principally different from those leading to SO_2 sensitivity, as *L. pulmonaria* is a markedly SO_2 sensitive species. The more Mn sensitive *H. physodes* is moderately SO_2 sensitive, whereas *L. conizaeoides* is tolerant to both Mn and SO_2.

1. Introduction

The abundance of epiphytic lichens in coniferous forests of Europe and North America has been found to be limited by high concentrations of Mn (HAUCK 2003). The main source of Mn in forest ecosystems is the soil, from which Mn is taken up by the tree roots and then translocated to all living tissues including the foliage and stem tissues that are designated to become bark soon (LÖVESTAM et al. 1990, LIN et al. 1996, HAUCK et al. 2002a). Immobilization of Mn in the bark is apparently a mechanism to detoxify excess amounts of Mn in trees (SCHMULL & HAUCK 2003a). Being very mobile in plant tissues, Mn is readily leached from foliage and bark (LEVIA & HERWITZ 2000, SCHMULL & HAUCK 2003b). Mn concentrations in both bark and stemflow affect epiphytic lichen performance (HAUCK et al. 2001b, 2002a, SCHMULL et al. 2002, SCHMULL & HAUCK 2003a, HAUCK 2005). As Mn is a natural constituent of the soil, Mn is apparently an important natural site factor for epiphytic lichens in coniferous forests, which has been overlooked until recently.

Field observations of Mn toxicity are supported by extensive experimental evidence, which has been obtained with the foliose lichen *Hypogymnia physodes*, which was selected as a model organism (HAUCK et al. 2002c, d, 2003, PAUL et al. 2003, 2004). Detrimental effects of Mn detected in these studies are, e.g., chlorophyll and chloroplast degradation, inhibition of soredia growth and photobiont propagation, degradation of mesosome-like structures and concentric bodies as well as swelling and shortening of fungal hyphae. The crustose lichen *Lecanora conizaeoides* was found to be indifferent to Mn supply in the field (HAUCK et al. 2001b) and in the laboratory (HAUCK et al. 2003) due to effective immobilization of Mn within the mycobiont cells (PAUL et al. 2003). While *H. physodes* and *L. conizaeoides* are chlorolichens with a *Trebouxia* photobiont, Mn uptake and toxicity in epiphytic cyanolichens has been studied in three selected species by HAUCK et al. (2005). This study revealed that Mn uptake increased and the effective quantum yield of photosystem 2 (PS2) was increasingly reduced from the tripartite, heteromerous cyanolichen *Lobaria pulmonaria* via the bipartite,

heteromerous cyanolichen *Nephroma helveticum* to the bipartite, homoimerous cyanolichen *Leptogium saturninum*.

The present study provides a direct comparison of the sensitivity of the PS2 to high Mn concentrations between chloro- and cyano-lichens. The aim was to test the hypothesis that cyanolichens are more sensitive to high Mn concentrations than chlorolichens. This hypothesis was based on field observations from the Pacific Northwest of North America, where conifer bark with relatively high Mn concentrations was only inhabited by chlorolichens, whereas bark with low Mn concentrations was colonized by all kinds of epiphytic lichens including bipartite cyanolichens (GOWARD & ARSENAULT 2000, HAUCK & SPRIBILLE 2002). The tripartite *Lobaria pulmonaria* with the green-algal primary photobiont *Dictyochloropsis* and the blue-green secondary photobiont *Nostoc* was found on conifer bark with low and medium-high Mn concentrations (HAUCK & SPRIBILLE 2002). Based on these field observations and the experiments of HAUCK et al. (2002c, d, 2003, 2005) and PAUL et al. (2003, 2004), the above hypothesis was specified to the hypothesis that the sensitivity of PS2 to high Mn supply increases in the order *Lecanora conizaeoides* < *Hypogymnia physodes* < *Lobaria pulmonaria* < *Nephroma helveticum* < *Leptogium saturninum*. The latter species stands for a gelatinous, homoiomerous, bipartite cyanolichen, while *N. helveticum* is a foliose, heteromerous, bipartite cyanolichen species. The ability of the PS2 to be photosynthetically active was indirectly assessed by means of fluorescence microscopy. This method, which gives evidence of the spatial distribution of photosynthetically active cells, is generally qualitative, but quantitative results were obtained with the help of a computer program, which was used to quantify the intensity of the fluorescence signal after conversion of digital images into grayscale pictures. The intensity of the fluorescence signal is taken as measure for the efficiency of PS2, as signal intensity is supposed to be proportional to the effective quantum yield of PS2 and gives, thereby, an estimation of the photosynthetic activity.

2. Materials and Methods

2.1. Lichen sampling and incubation procedure

Thalli of *Leptogium saturninum* (Dickson) Nyl., *Lobaria pulmonaria* (L.) Hoffm. and *Nephroma helveticum* Ach. were sampled from conifer bark in British Columbia, Canada. Samples of *L. saturninum* and *N. helveticum* were taken in Wells-Gray Provincial Park, ca. 25 km N Clearwater, 51°49' N, 120°07' W, 820 m, those of *L. pulmonaria* in Spahats Provincial Park, N Clearwater, 51°45' N, 120°00' W, 760 m. *Hypogymnia physodes* (L.) Nyl. was collected in the Harz Mountains, Germany (Rotes Bruch near Benneckenstein, 52°4' N, 10°43' E). *Lecanora conizaeoides* Nyl. ex Crombie was sampled in the New Botanical Garden of Göttingen, Germany (51°33' N, 9°57' E). The primary photobiont of *H. physodes* and *L. conizaeoides* is *Trebouxia* (Helms, Hauck & Friedl unpubl.), that of *L. pulmonaria Dictyochloropsis* and that of *L. saturninum* and *N. helveticum* is *Nostoc*. The latter is also the secondary photobiont in *L. pulmonaria* (RIKKINEN 2002). Air-dry thalli were stored in the dark at room temperature for a few days subsequent to collection (and during transport by aircraft in the case of the cyanolichens) and were then frozen at -30 °C.

Thallus pieces of up to 5 cm^2 with a vital appearance were preincubated on moist filter paper in Petri dishes at 80 % relative humidity, a day temperature (for 13 hours daily) of 13 °C during a photon flux of 15 µmol m^{-2} s^{-1}, and a night temperature of 10 °C for 1 week. After preincubation, thallus pieces were exposed to 25 ml of 10 mM $MnCl_2$ or deionized water for 1 h. All solutions were adjusted to pH 5 with NaOH and HCl. Subsequent to the exposure to metal solution or water, samples were briefly washed in deionized water and then placed on moist filter paper for 32 days at climatic conditions as described above. By this long recovery period, long-term reactions of the lichen thallus to excess Mn should be investigated rather than ecologically less relevant short-term responses.

2.2. Fluorescence microscopy and image analysis

Hand-cut cross-sections of lichen thalli were made immediately prior to fluorescence microscopy. This was sufficient, as section thickness is insignificant in the applied epi-illumination fluorescence microscopy (MODENESI et al. 1990).

Samples were studied with a Zeiss Axioskop microscope equipped with epi-fluorescence objectives (Carl Zeiss, Jena, Germany). Zeiss filters (BP 450 - 490, FT 510, LP 520) were used to isolate the excitation and the emission wavelengths. The BP 450 - 490 was used to isolate the 450 - 490 nm peak area of the high-pressure mercury lamp output for excitation. With help of the beam splitter FT 510, light of the excitation wavelength is reflected to the sample, whereas light of a wavelength > 510 nm passes to the emissionfilter. In addition, the emission filter LP 520 limits the light spectrum that can pass to the ocular to > 520 nm. Images were taken with a Nicon Coolpix 990 digital camera mounted on the microscope tube. Contrast and brightness control were held constant throughout the experiments. Signals were acquired, digitized and stored as images. The experiment was carried out in ten replicates.

Image files were converted to grayscale picture with Adobe Photoshop 7.0 software. The intensity of the fluorescence signal was quantified by calculating mean brightness values of the pictures. Brightness was expressed with the help of grayscale values ranging from 0 (black) to 255 (white). Determination of these values was carried out with Scion Image Beta 4.0.2 software (Scion Cooperation, Frederick, Maryland, U.S.A.). Five measurements per replicate sample were made in an area of the fluorescence image with high photobiont concentration. The size of the area, which was used to calculate a mean brightness value, depended on the distribution of photobiont cells within the thallus section, i.e. on the size of the photobiont layer in heteromerous lichens and on the thallus form in homoiomerous species. It amounted to 4.5 ± 0.8 cm^2 in *H. physodes*, 2.7 ± 0.7 cm^2 in *L. conizaeoides*, 7.3 ± 0.9 cm^2 in *L. saturninum*, 2.6 ± 0.5 cm^2 in *L. pulmonaria*, and 2.4 ± 0.3 cm^2 in *N. helveticum*. Brightness values in Table 11-1 are arithmetic means of the mean brightness values calculated from the five replicate measurements of every cross-section.

2.3. Statistics

Statistical significance of differences between mean values of Mn treated an untreated samples was calculated with Student's *t*-test after testing data for normal distribution with the Shapiro-Wilk test. Statistical analyses were carried out with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, U.S.A.).

3. Results

Reduction of the activity of PS2 due to $MnCl_2$ was most obvious in *L. saturninum* (Fig. 11-1). However, sensitivity was strongly variable between individual samples. While the *Nostoc* cyanobionts of some $MnCl_2$-exposed samples of *L. saturninum* revealed a red fluorescence signal in all parts of the thallus, other samples were devoid of any signal (Fig. 11-2). In other samples, only a part of the cyanobacteria was able to produce a fluorescence signal. The obvious effect of $MnCl_2$ on the intensity of the fluorescence signal, which can be taken from Figs. 11-1 and 11-2, is also supported by Table 11-1. Mean brightness values of the grayscale-converted fluorescence images were significantly different between $MnCl_2$-treated and control samples. Significantly different brightness values also occurred in *H. physodes* (Table 11-1), though these differences are hardly (or not) visually perceptible on the fluorescence images (Fig. 11-3). Considerable variation was observed in the intensity of the fluorescence signal in $MnCl_2$-treated samples of *N. helveticum* (Fig. 11-4), but a trend towards higher brightness values in Table 11-1 (standing for less intense red fluorescence as in *H. physodes*) was statistically insignificant. In both *L. pulmonaria* (Fig. 11-5) and *L. conizaeoides* (Fig. 11-6), the fluorescence signal remained unaffected by the $MnCl_2$ treatment (Table 11-1).

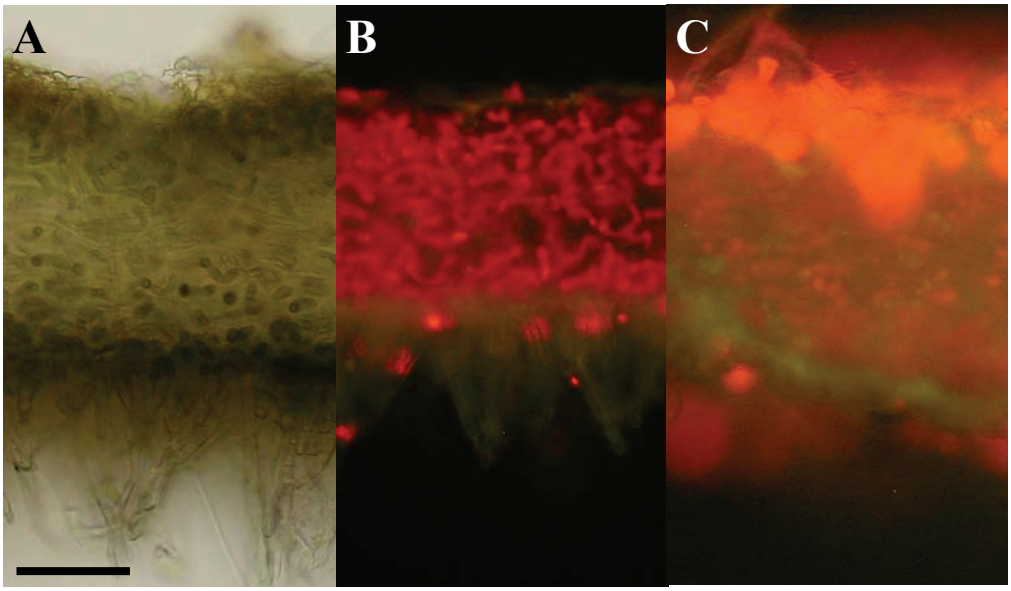

FIG. 11-1. Section through thallus of *Leptogium saturninum* incubated for 1 h with deionized water (A, B) or 10 mM $MnCl_2$ (C) and subsequently stored in the growth chamber for 32 days. A. Light microscopy image. B, C. Fluorescence microscopy image with intense red color indicating high chlorophyll fluorescence. Scale: 50 µm.

TABLE 11-1. Brightness values for chlorophyll fluorescence signals in fluorescence images converted to grayscale for green and blue-green lichen photobionts after exposure of lichen thallus pieces to $MnCl_2$ or deionized water

Species	Control	10 mM $MnCl_2$	P
Hypogymnia physodes	138 ± 2	146 ± 3	*
Lecanora conizaeoides	122 ± 3	115 ± 4	
Leptogium saturninum	178 ± 4	156 ± 8	*
Lobaria pulmonaria	115 ± 2	114 ± 3	
Nephroma helveticum	149 ± 8	155 ± 7	

Grayscale values ranging from 0 (black) to 255 (white). Arithmetic means ± standard error calculated from five replicate measurements in the photobiont layer (heteromerous lichens) or in photobiont-rich thallus parts (homoiomerous lichens) of 10 replicates per lichen species and $MnCl_2$ variant. Statistics: *t*-test (* $P \leq 0.05$).

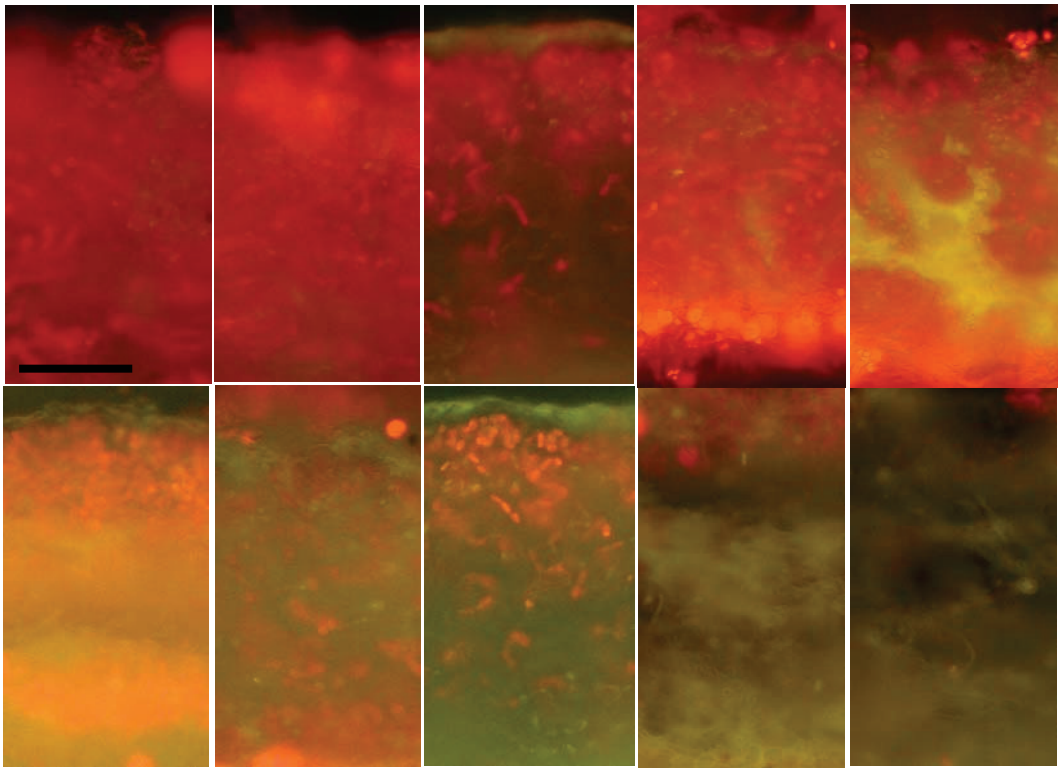

FIG. 11-2. Variation of chlorophyll fluorescence in thallus sections of *Leptogium saturninum* incubated for 1 h with 10 mM $MnCl_2$ and subsequently stored in the growth chamber for 32 days. Intense red color indicates high fluorescence. Pictures represent ten replicate samples. Scale: 50 µm.

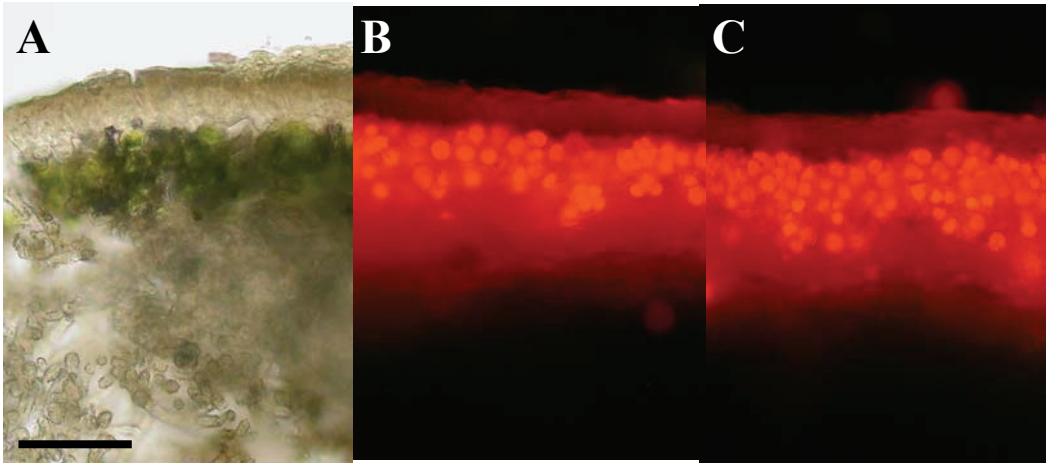

FIG. 11-3. Section through thallus of *Hypogymnia physodes* incubated for 1 h with deionized water (A, B) or 10 mM $MnCl_2$ (C) and subsequently stored in the growth chamber for 32 days. A. Light microscopy image. B, C. Fluorescence microscopy image with intense red color indicating high chlorophyll fluorescence. Scale: 50 µm.

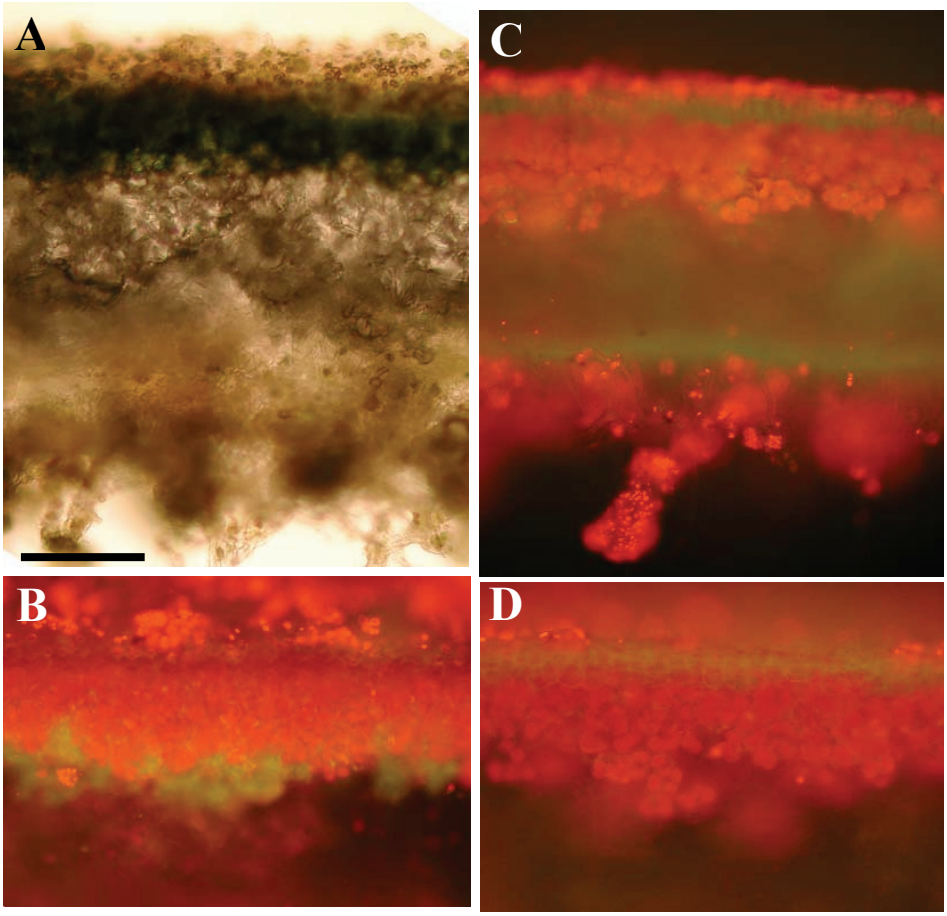

FIG. 11-4. Section through thallus of *Nephroma helveticum* incubated for 1 h with deionized water (A, B) or 10 mM $MnCl_2$ (C, D) and subsequently stored in the growth chamber for 32 days. A. Light microscopy image. B-D. Fluorescence microscopy image with intense red color indicating high chlorophyll fluorescence. Mn-treated samples show considerable variation in chlorophyll fluorescence (C, D). Scale: 50 µm.

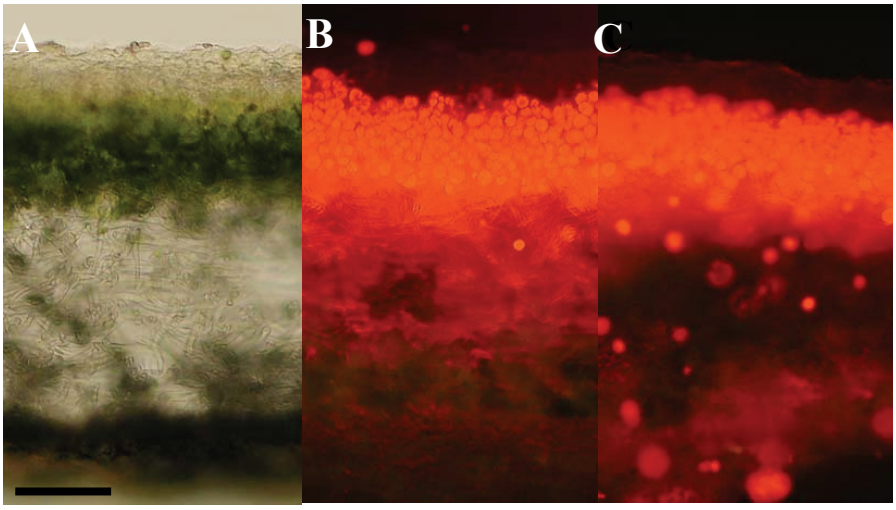

FIG. 11-5. Section through thallus of *Lobaria pulmonaria* incubated for 1 h with deionized water (A, B) or 10 mM $MnCl_2$ (C) and subsequently stored in the growth chamber for 32 days. A. Light microscopy image. B, C. Fluorescence microscopy image with intense red color indicating high chlorophyll fluorescence. Scale: 50 µm.

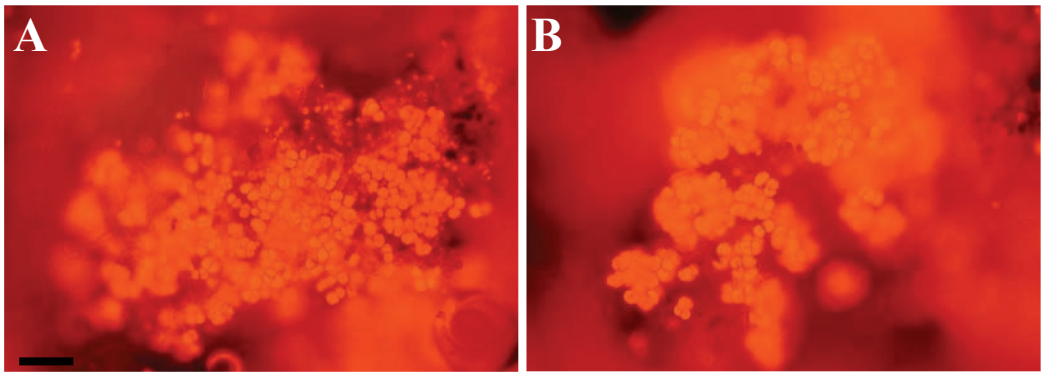

FIG. 11-6. View on thallus of *Lecanora conizaeoides* incubated for 1 h with deionized water (A) or 10 mM $MnCl_2$ (B) and subsequently stored in the growth chamber for 32 days. Fluorescence microscopy image with intense red color indicating high chlorophyll fluorescence. Scale: 50 µm.

4. Discussion

Fluorescence microscopy showed that the five studied lichen species react quite differently to the exposure to $MnCl_2$. In three species, more or less severe reduction of the fluorescence signal was observed. In the bipartite cyanolichen species, sensitivity to $MnCl_2$ was highly variable between replicate specimens of the same species. This suggests that the ability to withstand critical doses of Mn probably depends on the fitness of the individual lichen thallus. This matches well with results from field studies showing decreasing cover values of epiphytic lichen species with increasing Mn concentrations in bark or stemflow (HAUCK et al. 2001b, 2002a, SCHMULL et al. 2002, HAUCK 2003, SCHMULL & HAUCK 2003a). In all cases, where a detrimental influence of Mn on epiphytic lichen performance was suggested by correlation analysis, the abundance of sensitive lichen species decreased gradually with increasing Mn concentrations. Sensitive species were absent from microsites exceeding a certain critical Mn level (HAUCK 2000), but were just reduced in their abundance at microsites with medium-high Mn levels.

The high sensitivity of the PS2 in *L. saturninum* to $MnCl_2$ can at least partly be attributed to high rates for extracellular adsorption and intracellular uptake of Mn from $MnCl_2$ solution found in this species by HAUCK et al. (2005). *N. helveticum* took up less

Mn than *L. saturninum* from solution (HAUCK et al. 2005) and exhibited, thus, a less severe reduction of the fluorescence signal. High variability is the cause of lacking statistical significance for differences in the fluorescence signal in *N. helveticum*. The different uptake rates are the most probable explanation for the different sensitivity of the PS2 to $MnCl_2$ in *L. saturninum* and *N. helveticum*, as *Nostoc* is the photobiont in either lichen species (RIKKINEN 2002). The higher uptake in *L. saturninum* than in *N. helveticum* is probably caused by the different thallus structure. To test this hypothesis, Mn uptake characteristics of homoiomerous, gelatinous versus heteromerous cyano-lichens should be comparably studied on the cellular and subcellular level as done in *H. physodes* and *L. conizaeoides* by PAUL et al. (2003).

The PS2 in *L. pulmonaria* was apparently not affected by $MnCl_2$. Relative to *N. helveticum* and *L. saturninum*, this is plausible as *L. pulmonaria* takes up less Mn from $MnCl_2$ than *N. helveticum* and *L. saturninum* (HAUCK et al. 2005). This result matches well with the field observation of HAUCK & SPRIBILLE (2002) that *L. pulmonaria* colonized conifer bark with higher Mn concentrations than bipartite cyanolichens in the Pacific Northwest of North America. The lacking sensitivity of the PS2 to $MnCl_2$ in *L. pulmonaria* is interesting in comparison to the bipartite chlorolichen *H. physodes*, where $MnCl_2$ caused a significant change in fluorescence signal. This suggests that *H. physodes* is more sensitive to high Mn concentrations than *L. pulmonaria*. Since both species have a similar thallus structure, this could be due to a higher Mn tolerance in the green-algal photobiont of *L. pulmonaria*, *Dictyochloropsis*, than in the *Trebouxia* photobiont of *H. physodes*. Experimental data to test this hypothesis are lacking. Alternatively, *L. pulmonaria* could have a more effective mechanism for intrathalline Mn immobilization than *H. physodes*. The higher Mn tolerance in *L. pulmonaria* than in *H. physodes* proves that the mechanisms responsible for Mn toxicity in these lichen species differ from mechanisms causing SO_2 toxicity. This is because *L. pulmonaria* is known to be much more sensitive to SO_2 and its derivates formed in aqueous solution than *H. physodes* (TÜRK et al. 1974).

In contrast to *L. pulmonaria*, the lacking reaction of the fluorescence signal to $MnCl_2$ in *L. conizaeoides* comes up to expectations, as PAUL et al. (2003) showed that *L. conizaeoides* can maintain the concentrations of free intracellular Mn very effectively low by immobilization. This result also shows that the high sensitivity of *L. saturninum*

to Mn is not just due to being a homoiomerous lichen, because the thallus of *L. conizaeoides* is also homoiomerous, but that more detailed study is necessary to detect the causes of the high Mn uptake and sensitivity in *L. saturninum*.

In conclusion, the present results support the hypothesis that cyanolichens are generally more sensitive to high Mn concentrations than chlorolichens. However, the present results do not support the hypothesis that the tripartite *L. pulmonaria* has an intermediate position in terms of Mn sensitivity between the bipartite chlorolichens and the bipartite cyanolichens. Rather, Mn sensitivity of the PS2 increased in the order *L. conizaeoides*, *L. pulmonaria* < *H. physodes*, *N. helveticum* < *Leptogium saturninum*. The observation that the highly SO_2 sensitive *L. pulmonaria* is as tolerant to excess concentrations of Mn as the SO_2 tolerant *L. conizaeoides* shows that mechanisms leading to Mn toxicity in lichens are principally different from those leading to SO_2, or more exactly S(IV), toxicity.

Chapter 12

Manganese as a site factor for epiphytic lichens

Abstract

Decreasing abundance of epiphytic lichens with increasing Mn supply from the substrate or from stemflow was found in several coniferous forests of Europe (Germany) as well as western (Montana, British Columbia) and eastern North America (New York State). Experiments carried out with *Hypogymnia physodes* and other species of chloro- and cyano-lichens suggests that these correlations are causal. High Mn concentrations, e.g., reduce chlorophyll concentrations, chlorophyll fluorescence and degrade the chloroplast in lichen photobionts. Excess Mn inhibits the growth of soredia of *H. physodes* and causes damage in the fine- and ultra-structure of the soredia. Adult lichen thalli remain structurally unaffected by Mn. Mn uptake does not result in membrane damage. Ca, Mg, Fe and perhaps also Si alleviate Mn toxicity symptoms in *H. physodes*. *Lecanora conizaeoides* is not sensitive to Mn both in laboratory experiments and in the field. The data suggest that high Mn concentrations are an important site factor for epiphytic lichens in coniferous forests that has been overlooked until recently. Mn reaching the microhabitat of epiphytic lichens is primarily soil-borne and is usually not derived from pollution.

1. Introduction

The significance of cation concentrations for the physiology and distribution of vascular plants is widely accepted and has been subject to extensive study. Exhaustive knowledge is available, e.g., for effects of Ca, Mg, Na, Fe, Al, Mn, Zn, Cu and a range of non-essential heavy metals. Elemental analyses of the soil are routinely carried out with standardized extraction methods to describe the chemical environment of a vascular plant. The situation is quite different in lichen ecology. Here, scientists occasionally even discuss which principal environmental sources contribute to the cation supply of lichens. SCHADE (1970) and RASMUSSEN et al. (1980), for instance, denied any cation uptake from the substrate. Nevertheless, there is conclusive evidence from several field studies that lichens take up cations from air-borne particles, precipitation and the substrate (NASH 1989; FARMER et al. 1991; GAUSLAA et al. 1998; HAUCK 2003).

Detailed investigations into the pathways cations take into the lichen thallus or of the fate of cations within the thallus primarily deal with heavy metals. Based on correlations between high Mn concentrations and low epiphytic lichen abundance repeatedly found in the field, we conducted a case study of the ecophysiological role of this transition metal in lichens. Our research on effects of Mn on lichens differs principally from previous heavy metal and lichen studies of, e.g., NIEBOER et al. (1972), NASH (1975), PUCKETT (1976), BROWN & BECKETT (1983), BECKETT & BROWN (1984a), PURVIS & HALLS 1996, or GARTY et al. (1992). This is because the cited studies generally deal with transition metals that either occur in extreme heavy metal-rich rock environments or that are found in "normal" ecosystems due to anthropogenic pollution. In the case of Mn toxicity, we are talking about toxic concentrations that naturally occur in forest ecosystems.

2. Biological function of manganese in lichens

Any study dealing with toxicity effects of Mn should consider that Mn also is an essential micronutrient. Mn participates in many basic vital processes, e.g., in the water-splitting reaction of photosynthesis or in translation (BURNELL 1988; CROWLEY et al.

2000). Further, Mn is a constituent of many enzymes, for instance, of several dehydrogenases, transferases and hydrolases. The Mn-containing enzymes isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase take part in the citrate cycle. Mn-dependent superoxide dismutase occurs in every lichen species (WHITTAKER 2000; STROUPE et al. 2001). These examples show that lichens are not generally limited by Mn, but only by excess concentrations. However, the concentration of Mn required by a lichen in the field is apparently very low, as a limitation of lichen abundance due to Mn deficiency has never been found, so far. Culture media used for the cultivation of lichen bionts, such as Bold's Basal Medium, contain only some µM of Mn (AHMADJIAN 1993).

3. Field data

Field observations on correlation between epiphytic lichen abundance and Mn supply were made in several coniferous forests of central Europe as well as western and eastern North America. Decreasing cover values of the foliose chlorolichen *Hypogymnia physodes* and other lichens with increasing Mn concentration in bark or stemflow were repeatedly found in stands of *Picea abies* in the Harz Mountains, Germany (HAUCK 2000, 2003; HAUCK et al. 2001b, 2002a). In one spruce stand, cover of *H. physodes* was most closely correlated with the ratio of Mn to Ca concentrations in stemflow (HAUCK et al. 2002a). In general, however, Mn concentrations in bark revealed more often an influence on lichen diversity than the Mn content of stemflow (HAUCK 2003, 2005). Cover of the crustose chlorolichen *Lecanora conizaeoides*, which is known for its toxitolerance, did not respond to Mn concentrations. A wide range of chemical site factors was analyzed in these investigations so that intercorrelation with another ion concentration could be ruled out as the cause of the correlations between lichen abundance and Mn concentrations (HAUCK et al. 2001b, 2002a, b; HAUCK & RUNGE 2002).

In a montane *Picea rubens-Abies balsamea* forest of the Adirondacks, upstate New York, chlorolichen species including *Hypogymnia physodes* decreased with increasing Mn/Fe ratio in *P. rubens* bark (SCHMULL & HAUCK 2003a), while a few lichen species decreased with the Mn content of stemflow (SCHMULL et al. 2002). In *Picea engelmannii-Abies lasiocarpa* stands of the North American Intermountain West (in

northwestern Montana) cover of several epiphytic chlorolichens decreased with increasing Mn concentration of the substrate or its ratio to Ca, Mg or Fe (HAUCK 2005). While epiphytic cyanolichens were absent from all studies sites mentioned, so far, observations on *Pinus monticola* growing in the dripzone of *Populus balsamifera* in northwestern Montana indicated that cyanolichens could be more sensitive to high Mn supply than chlorolichens. Bipartite cyanolichens of the genera *Leptogium*, *Lobaria* and *Nephroma* were restricted to microhabitats with the lowest Mn content of bark, i.e., to pine branches within the dripzone of *Populus* (HAUCK & SPRIBILLE 2002). The tripartite lichen *Lobaria pulmonaria*, which contains the green alga *Dictyochloropsis* and the cyanobacterium *Nostoc* (RIKKINEN 2002) grew on bark of medium Mn content. The most Mn-rich microhabitats were only inhabited by chlorolichens. These observations on cyanolichens by HAUCK & SPRIBILLE (2002) are based on a very small data set. However, similar findings were made by GOWARD & ARSENAULT (2000) on *Picea engelmannii* × *glauca* in British Columbia before, though these authors did not notice the correlation with Mn concentrations in their data.

4. Biogeochemical cycling of manganese in forest ecosystems

Mn in tree bark is primarily soil-borne. Solubility strongly increases with increasing acidity. Further, it is increased due to water-logging, because insoluble Mn^{3+} and Mn^{4+} ions are converted into soluble Mn^{2+} in a reducing environment (NORVELL 1988). As an essential micronutrient, Mn^{2+} is readily taken up by the roots of vascular plants (CLARKSON 1988). With the transpiration flux, Mn^{2+} ions reach the stem and are allocated to all living tissues, and the young foliage in particular (LIN et al. 1996). Albeit its essential metabolic role, excess concentrations of Mn are toxic to vascular plants (EL-JAOUL & COX 1998). This includes coniferous trees, where exposure to high Mn concentrations results in damage related to reduced uptake of Fe, Ca and Mg (KEIL et al. 1986; GARTNER et al. 1990; KAUS & WILD 1997). Translocation of Mn to the bark is apparently a widespread mechanism in trees to avoid toxic concentrations of free Mn^{2+} in physiological more important tissues. LÖVESTAM et al. (1990) and SLOOF & WOLTERBEEK (1993) found evidence for the radial transport of Mn through the xylem parenchyma in *Picea abies* and *Populus* spec. Mn is often deposited in inner layers of the bark, which was taken as additional evidence for the mainly soil-borne (and not

atmospheric) origin of the ions (LÖVESTAM et al. 1990). *Abies balsamea* from upstate New York, however, was found to deposit Mn primarily in the outer bark, though comparison of element concentrations in bark, soil, stemflow and incident precipitation indicated that the bark Mn content was most significantly affected by the concentration available from the soil (SCHMULL et al. 2002; SCHMULL & HAUCK 2003a). FINK (1999) provided detailed information of Mn deposition in the inner bark of *Malus domestica*, where cells with high Mn concentration become necrotic, while surrounding parenchyma cells undergo repeated cell divisions and, finally, form a periderm that seals the areas with Mn deposits. Other species deposit Mn in lignifying tissues before their death. *Abies balsamea* immobilizes Mn in crystals in spongy cork and sclerotic phelloid cells of the outer bark (SCHMULL & HAUCK 2003a). The chemical composition of these crystals is not known, but the absence of significant amounts of P, S, and Cl suggests that oxalate could be the corresponding anion. This is supported by Ca oxalate crystals, which are known to be formed in the bark of *Picea abies* under conditions of Ca profusion (SCHMIDT-VOGT 1986), and by the fact that Mn is deposited in oxalate crystals in the epiphytic lichen *Hypogymnia physodes* (PAUL et al. 2003) and the saxicolous lichen *Pertusaria corallina* (WILSON & JONES 1984).

While the disposal of Mn in dying bark tissues is economic for the tree, it entails that those part of a tree, which is inhabited by epiphytes, namely the bark surface, is particularly rich in Mn. Mn^{2+} is a very mobile ion in plant tissues and, thus, together with K^{+}, readily leached from the foliage and from bark (FASSBENDER 1977; SCHMIDT 1987). Leaching of Mn strongly depends on weather conditions. LEVIA & HERWITZ (2000) found the most intense Mn leaching from *Carya glabra* during snowmelt at temperatures around 0 °C, when the viscosity of stemflow and the surface tension were particularly high. This matches with observations of marked peaks of the Mn content of stemflow during the cold season in stands of *Picea abies* in the Harz Mountains, which could not be attributed to peak concentrations in incident precipitation (HAUCK 2000). Thus, snowmelt water and stemflow from rainfall at low temperature considerably contribute to the Mn budget of bark surfaces, although the amount of such stemflow is negligible compared to stemflow that occurs in the warm season in temperate and boreal forests (HAUCK 2000; LEVIA & UNDERWOOD 2004).

As a consequence of their high mobility, Mn^{2+} ions are also readily (re-)absorbed by tree canopies. Most of the absorbed Mn remains on the canopy surface, i.e., in the foliage (especially in epicuticular wax layers) or in the bark (LIN et al. 1995, LIN & SCHUEPP 1996). Mn absorbed in the canopy is partly derived from precipitation and partly from aerosols (SCHMIDT 1987), whereas some ions are re-absorbed after leaching.

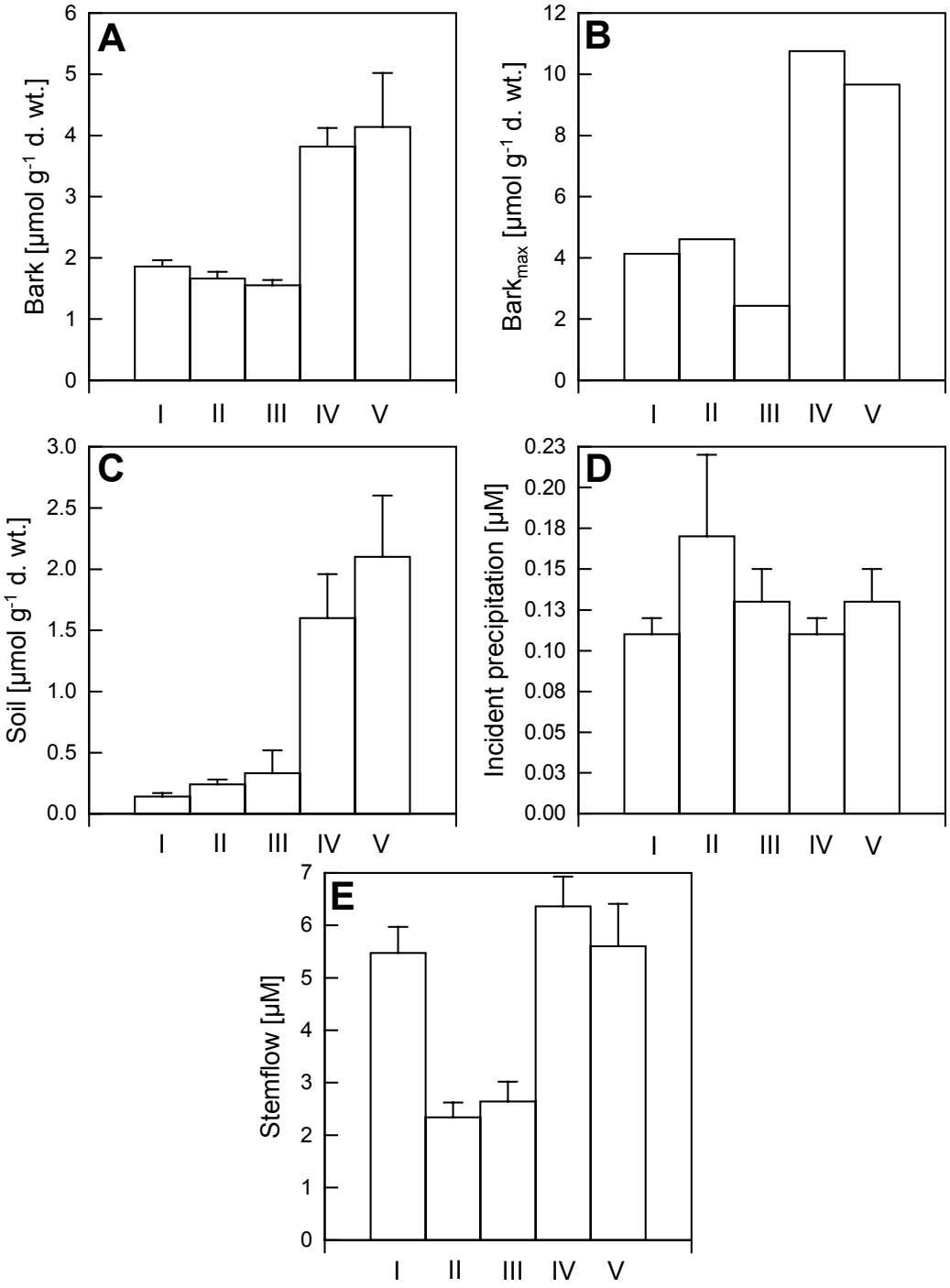

FIG. 12-1. Mn concentrations in five mature *Picea abies* stands of the Harz Mountains, Germany. A. Mean total concentration in bark. B. Maximum total concentration in bark. C. Concentration in soil extractable with 100 mM EDTA. D. Total concentration in incident precipitation. E. Total concentration in stemflow. Error bars indicate standard error. Study sites: I, Acker-Bruchberg, 790 m, healthy trees (HAUCK 2000; HAUCK et al. 2001b; HAUCK & RUNGE 2002); II, Acker-Bruchberg, 820 m, trees partly affected by pollution-caused forest dieback (HAUCK 2000; HAUCK et al. 2001b; HAUCK & RUNGE 2002); III, Mt. Brocken, 1000 m, trees partly dieback-affected (HAUCK et al. 2002b); IV, Acker-Bruchberg, 790 m, healthy trees (HAUCK 2000); V, Rotes Bruch, 550 m, trees partly dieback-affected (HAUCK et al. 2002b). For sampling design and analytical methods confer the cited work.

Quantifications for the latter process have not been published. Atmospheric deposition of Mn is generally of lower significance for the Mn content of bark than the amounts that are deposited in a tree after root uptake. This is supported by the close correlation of the Mn content of bark with the Mn content of the soil, but not of incident precipitation, which was found in five *Picea abies* stands of the Harz Mountains (Fig. 12-1A–D).

Stemflow concentration is more difficult to predict (Fig. 12-1E), as it seems to depend on several variables, such as the Mn content of soil, incident precipitation and dry deposition and on the physiological condition of the trees. Since the bark Mn content has usually a more significant effect on lichen abundance than the Mn content of stemflow and since, moreover, the deposition of Mn from the atmosphere is primarily of natural origin in many areas, these data suggest that any effects of Mn on lichen vegetation are generally natural, but might be enhanced due to anthropogenic soil acidification. Canada is an exception from this rule, because the Mn-containing compound methylcyclopentienyl manganese tricarbonyl (MMT) is used since 1977 as an antiknock additive of gasoline on a large scale (LIN et al. 1995). However, even there the quantitative role of anthropogenic Mn emissions on the total atmospheric Mn input is controversial (FORGET et al. 1994; ZAYED et al. 1999). In our own measurements of Mn concentrations in incident precipitation from northern New York State and northwestern Montana, not far from the Canada border (SCHMULL et al. 2002), we did not find significantly higher concentrations than in Germany, where MMT is not used (HAUCK & RUNGE 2002; HAUCK et al. 2002a, b).

5. *In vitro* Manganese uptake in lichens

Uptake of Mn from $MnCl_2$ solutions has been studied in the chlorolichens *Hypogymnia physodes*, *Lecanora conizaeoides* (HAUCK et al. 2002c), *Alectoria sarmentosa*, *Bryoria fuscescens* (Chapter 8), in the tripartite lichen *Lobaria pulmonaria* as well as in the bipartite cyanolichens *Leptogium saturninum* and *Nephroma helveticum* (Chapter 9). Both extracellular adsorption and intracellular uptake of Mn is commensurate with the concentration applied with the incubation medium (Table 12-1). At a given Mn concentration, extracellular adsorption and intracellular uptake reach a saturation level

after 10–20 min. Rates of extracellular adsorption are generally much higher than of intracellular uptake (HAUCK et al. 2002c). Water-soluble, apoplastic Mn is quantitatively insignificant even after incubation with $MnCl_2$ concentrations as high as 100 mM. All of these results agree well with previous studies of the uptake of transition metals in lichens (NIEBOER et al. 1978; NASH 1989; BROWN & BROWN 1991; RICHARDSON 1995). The spatial pattern of Mn in the thallus with significant concentrations at extracellular exchange sites and intracellularly is a result of the physicochemical properties of Mn^{2+}, which has been classified as a borderline ion with strong class A character by NIEBOER & RICHARDSON (1980).

TABLE 12-1. Concentrations of extracellularly bound and intracellular Mn (in µmol g^{-1} d. wt.) in *Alectoria sarmentosa* and *Bryoria fuscescens* subsequent to 1 h-incubation with metal solutions containing $MnCl_2$

Incubation [mM]	Alectoria sarmentosa		Bryoria fuscescens	
	Extracellularly bound	Intracellular	Extracellularly bound	Intracellular
Control	1.36 ± 0.13 a	0.35 ± 0.06 a	2.12 ± 0.29 a	0.63 ± 0.16 a
Mn 0.1	2.60 ± 2.29 a	0.53 ± 0.03 a	2.51 ± 0.08 a	0.90 ± 0.13 a
Mn 1	13.0 ± 2.2 b	1.38 ± 0.15 b	16.5 ± 0.4 b	1.84 ± 0.08 a
Mn 100	49.6 ± 2.4 c	3.88 ± 0.30 c	80.1 ± 5.9 c	9.68 ± 1.24 b

Arithmetic means ± standard error. Within a column, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 10$). Controls run with deionized water.

While the aquatic green alga *Scenedesmus subspicatus* is known to convert significant proportions of extracellular Mn^{2+} into Mn^{3+} and Mn^{4+} (KNAUER et al. 1999), only a small percentage of the total extracellular Mn is Mn^{3+} or Mn^{4+}. This concerned 6 % of the total Mn in untreated samples of *Hypogymnia physodes* and 16 % of the total Mn in *Lecanora conizaeoides* bound at extracellular exchange sites (HAUCK et al. 2002*c*). Extracellular cation exchange sites that bind to Mn are not only found on the surface of cell walls (RICHARDSON 1995), but also in extracellular polysaccharide matrices. In *H. physodes*, as much Mn was found in the interhyphal matrix as in the cell walls of cortical hyphae (PAUL et al. 2003). Similar results were obtained from experiments with the gelatinous cyanolichen *Leptogium saturninum*, where the *Nostoc* cyanobiont forms extensive exopolysaccharide matrices (Paul & Hauck unpubl.).

Photobiont cell walls in thallus pieces of *H. physodes* incubated with 5 mM $MnCl_2$ for 1 h contained about 20 % of the concentration of Mn found in the fungal cell walls (PAUL et al. 2003). The Mn concentration in the algal cell lumina was less than 10 % that of the fungal cell interior. In soredia of *H. physodes* cultured on agar plates with BBM and varying $MnCl_2$ concentrations for 8 days, neither extracellular nor intracellular Mn concentrations differed between the fungus and the alga (HAUCK et al. 2002d). This suggests that the heteromerous thallus structure of *H. physodes* enables the mycobiont of adult thalli to protect the photobiont from invasion by Mn to a certain extent. *H. physodes* immobilizes excess amounts of Mn in intracellular polyphosphate bodies. This has been found in fungal and algal cells of soredia and in cortical hyphae of fully developed thalli (PAUL et al. 2003). Such accumulation of Mn in polyphosphate bodies is widespread in fungi, algae and cyanobacteria (BAXTER & JENSEN 1980; TILLBERG et al. 1980; TURNAU et al. 1993). In soredia of *H. physodes*, extracellular encrustations of Mn phosphate (Fig. 12-2) are frequent (HAUCK et al. 2002d).

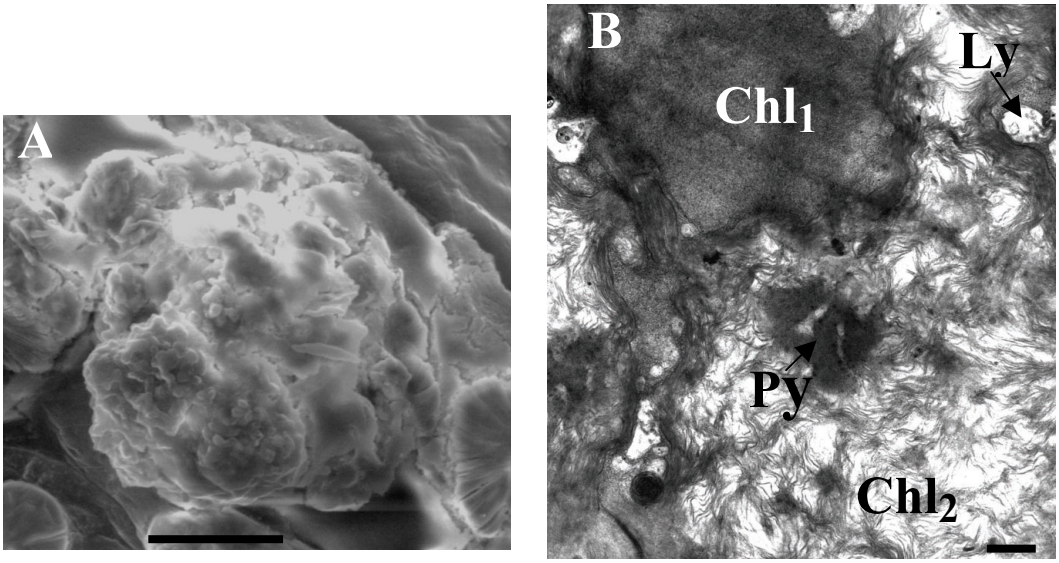

FIG. 12-2. A. Mn accumulation in extracellular phosphate encrustation in soredium of *Hypogymnia physodes* cultured on BBM with 7 mM $MnCl_2$ for 8 days. Scale: 5 µm. B. Ultrathin section of *Trebouxia* photobiont in soredium of *H. physodes* cultured on BBM with 500 µM $MnCl_2$ for 11 days. The picture shows areas, where the chloroplast is complety (Chl_1) or partly (Chl_2) degenerated. The central pyrenoid is reduced in size (Py). Lysosome-like vesicles (Ly) are involved in the break-down of organelles destroyed by Mn. Scale: 500 nm.

The immobilization of Mn in phosphate deposits does not lead to intracellular Mn deficiency as shown by analyzing adenine nucleotide concentrations (HAUCK et al. 2003). This is probably because of a very efficient phosphate uptake mechanism in lichens including *H. physodes* (FARRAR 1976; HYVÄRINEN & CRITTENDEN 1998).

6. Manganese uptake in the field

Mn uptake in the field is mostly from the substrate as correlation analyses show (BOSSERMAN & HAGNER 1981; DE BRUIN & HACKENITZ 1986; SLOOF & WOLTERBEEK 1993). In the Harz Mountains, HAUCK (2000) found a close correlation between the total Mn content of *H. physodes* and the inhabited *Picea abies* bark ($r = 0.84$, $P \leq 0.001$), but no significant correlation with the mean Mn concentration in stemflow. In another spruce stand of the Harz Mountains with above-average content of plant-available Mn in the soil, Mn concentrations from stemflow were found to be more significant that those in bark (HAUCK et al. 2002c). The availability of Mn from bark differs between tree species. While it is obvious from correlations between Mn concentrations and the abundance of epiphytic lichen species that Mn is readily available from spruce bark as shown for *Picea abies*, *P. engelmannii* and *P. rubens* (HAUCK et al. 2001b; SCHMULL & HAUCK 2003a; HAUCK 2005), Mn was found to be little available from the bark of *Abies balsamea* (SCHMULL & HAUCK 2003a). This is because of the above-mentioned crystals, *A. balsamea* forms in its bark. These crystals benefit not only the tree, but also its epiphytes. Though only studied in *A. balsamea*, so far, this result of SCHMULL & HAUCK (2003a) could be the clue to explain the general high abundance of epiphytic lichens on *Abies*. The availability of Mn from bark also differs between living and dead trees of the same tree species (SCHMULL & HAUCK 2003b).

The maximum depth, to which mycobiont hyphae penetrate the substrate is controversially discussed (ASCASO et al. 1980; ESTEVEZ et al. 1980), but certainly depends on the lichen species. Fungal hyphae even of an erhizinate species such as *H. physodes* are in close contact to the bark (Fig. 12-3). An uptake of Mn from the substrate by these hyphae in the presence of a minimum amount of water is easily conceivable. The uptake of Mn from stemflow is also plausible, although the stemflow volume is rather small in coniferous forests and the contact time of lichens with stem-

flow is limited. This is because of the relatively high binding affinity of Mn to the extracellular exchange sites in lichen thalli (NIEBOER & RICHARDSON 1980). Mn^{2+} ions efficiently replace Ca^{2+} and Mg^{2+} from these binding places (HAUCK et al. 2002c, d; PAUL et al. 2003) so that short times of exposure to Mn are sufficient for the extracellular adsorption of significant amounts of ions. In this respect, Mn and other transition metals differ principally from ions with weak binding affinity to the extracellular binding places.

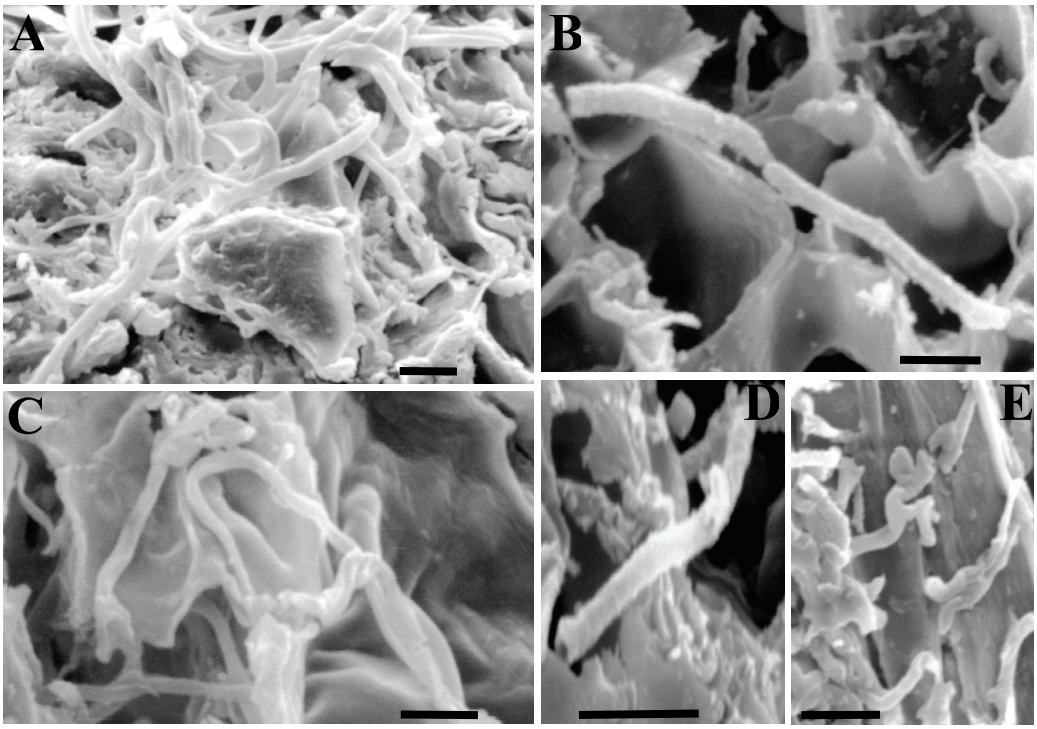

FIG. 12-3. Lichen/substrate interface of *Hypogymnia physodes* (A, B, D) and *Lecanora conizaeoides* (C, E) showing the close contact of mycobiont hyphae with bark and wood, which is the structural basis for cation uptake from the substrate. Substrates shown are *Picea abies* bark (A, C), *Quercus robur* bark (B, D) and conifer wood (E). Scale: 10 µm.

HAUCK & GROSS (2003) showed for K that concentrations found in *Picea abies* stemflow of the Harz Mountains were not high a enough to cause significant extracellular adsorption (or intracellular uptake) in *H. physodes* despite of an incubation time of 2 h, which is often not achieved by soaking with stemflow. In the case of Mn,

experiments with cyanolichens showed that ions adsorbed at extracellular binding places are transferred into cell long after the actual exposure of the lichen thallus to the source of Mn^{2+} ions (Chapter 9). This agrees with a study of BROWN & BECKETT (1985) of Cd uptake in *Cladonia portentosa* and *Peltigera horizontalis*. These results suggest that even short exposures to Mn^{2+} in stemflow, e.g., during a peak leaching event in the cold season, can be sufficient to affect epiphytic lichens. Unfortunately, our knowledge of the temporal and spatial dynamics of cation uptake in lichens under field conditions is still very limited. Exemplary measurements showed that epiphytic lichens growing on acidic bark contained between 0.3–1.6 µmol g^{-1} d. wt. intracellularly and around 1–2 µmol g^{-1} d. wt. at the extracellular exchange sites (Table 12-2). The bipartite cyanolichens *Leptogium saturninum* and *Nephroma helveticum* had a higher intracellular Mn content than the tripartite *Lobaria pulmonaria* or the bipartite chlorolichens (Table 12-2).

TABLE 12-2. Concentration of extracellularly adsorbed and intracellular Mn (in µmol g^{-1} d. wt.) in epiphytic lichens sampled from acidic bark in Europe and North America

Lichen species	Origin	Substrate	Intracellular	Extracellular
Alectoria sarmentosa	Sweden	*Picea abies*	0.4	1.4
Bryoria fuscescens	Sweden	*Picea abies*	0.6	2.1
Hypogymnia physodes	Germany	*Quercus robur*	0.6	1.5–1.6
Lecanora conizaeoides	Germany	*Picea abies*	0.7	1.1–1.3
Leptogium saturninum	Canada	*Picea engelmannii*	1.5	1.0
Lobaria pulmonaria	Canada	*Picea engelmannii*	0.3	0.9
Nephroma helveticum	Canada	*Abies lasiocarpa*	1.6	1.1

Arithmetic means from 5–8 replicate samples. Extracellularly adsorbed ions analyzed in 20 mM $NiCl_2$ extracts and intracellular ion measured in acid digests with 65 % HNO_3 by means of AAS.

7. Manganese related damage in lichens

When we obtained our first field data that suggested a possible limitation of epiphytic lichen abundance in coniferous forests by high, ambient Mn concentration only a few studies of lichens and heavy metals were published, where Mn was involved. BURTON et al. (1981) used Mn as an example for a hardly toxic transition metal and showed that significant K^+ losses in *Cladonia rangiferina* were induced by Mn only at a concentration as high as 1 M applied for 90 min. GOYAL & SEAWARD (1982) found the total thallus content of K in the heteromerous cyanolichen *Peltigera canina* to be reduced by 50–70 % after exposure to 2–16 mM $MnSO_4$ for 2 h. GARTY et al. (1992) found a

reduced chlorophyll content in *Ramalina lacera* after incubation with 20 mM $MnCl_2$ for 30 min, however, this experiment was conducted at pH 2.0 and the acidity alone similarly reduced the chlorophyll content compared to an untreated control as the combined treatment of acidity and $MnCl_2$. Against the background of this scarce evidence for Mn toxicity in lichens, we started experiments on Mn toxicity in epiphytic lichens occurring in coniferous forests of Europe and North America to test whether the correlations between Mn concentration and epiphytic lichen abundance from our study plots were causal.

In most lichens investigated, Mn strongly reduced chlorophyll concentrations and chlorophyll fluorescence. In *H. physodes* chlorophyll concentrations were reduced both in adult thalli and in soredia exposed to excess amounts of Mn (HAUCK et al. 2002d, 2003). A concentration of 1 mM $MnCl_2$ applied to thalli of *H. physodes* for 1 h reduced the chlorophyll content even more than an equimolar solution of $CuCl_2$ (HAUCK et al. 2003). Ultrastructural studies of *H. physodes* soredia exhibited that excess Mn leads to the degradation of the chloroplast (Fig. 12-2) of the *Trebouxia* photobiont (PAUL et al. 2004). Among the cyanolichens, Mn reduced chlorophyll fluorescence most in the homoiomerous, bipartite *Leptogium saturninum*, second most in the heteromerous, bipartite *Nephroma helveticum* and least in the tripartite *Lobaria pulmonaria*, which contains *Nostoc* in cephalodia (Chapter 9, 10). Remarkably, chlorophyll fluorescence was more affected in *H. physodes* than in *L. pulmonaria*, though the latter is much more sensitive to SO_2 and related compounds than the former (TÜRK et al. 1974). This clearly shows that different mechanisms lead to the partly similar toxicity symptoms induced by Mn and SO_2.

While structural damage due to Mn uptake has not been observed yet in adult lichen thalli (PAUL et al. 2003), various damage symptoms have been detected in soredia of *H. physodes* with scanning and transmission electron microscopy. SEM revealed strongly swollen and shortened mycobiont hyphae in the soredia (HAUCK et al. 2002d). While intact soredia grown on agar medium with only 7 µM $MnCl_2$ had a fine, densely branched network of hyphae that closely surrounded the photobiont, many hyphae in soredia cultured with excess amounts of $MnCl_2$ lost their contact to the *Trebouxia* photobiont. Since the fungal cell walls are the only water source for the photobiont (HONEGGER et al. 1996), this loss of contact must be fatal for the photobiont. This

agrees with the observation that many algal cells were smaller or collapsed in Mn treated soredia (HAUCK et al. 2002d). Ultrastructural studies showed that $MnCl_2$, further, reduced the quantity of mesosome-like structure in the mycobiont (PAUL et al. 2004). These organelles are supposed to be involved in the metabolite transfer from the photobiont to the fungus (PEVELING 1972, 1976; BOISSIÈRE 1982). Thus, surplus Mn apparently interrupts both the water and the metabolite transfer between the lichen bionts. Degradation of concentric bodies in soredia grown on Mn-rich substrate suggests that high Mn concentrations are also capable of reducing the drought resistance of lichens (PAUL et al. 2004), as concentric bodies are thought to be somehow involved in enabling the lichen fungus to withstand the repeated drying and rewetting cycles (PEVELING et al. 1985; HONEGGER 1995). Moreover, excess Mn inhibited the formation of *Trebouxia* autospores and, thereby, the propagation of the photobiont in *H. physodes* soredia (PAUL et al. 2004).

A constant accompaniment of Mn uptake in lichens is the release of basic cations, such as Ca and Mg (Table 12-3).

TABLE 12-3. Concentrations of free apoplastic, extracellularly bound and intracellular Ca, Mg and K (in µmol g^{-1} d. wt.) in *Alectoria sarmentosa* subsequent to 1 h-incubation with metal solutions containing $MnCl_2$ or $CuCl_2$

Incubation [mM]	Extracellular, water-soluble	Extracellularly bound	Intracellular
Ca content:			
Control	155 ± 148 (10) a	174 ± 169 (12) a	342 ± 166 (55) a
Mn 0.1	237 ± 231 (13) a	193 ± 186 (11) a	423 ± 204 (56) a
Mn 1	397 ± 160 (23) a	270 ± 179 (23) a	380 ± 133 (29) a
Mn 100	349 ± 191 (24) a	261 ± 226 (20) a	350 ± 179 (41) a
Cu 100	1.35 ± 0.25 (0) a	45.2 ± 5.3 (4) a	213 ± 5 (17) a
Mg content:			
Control	0.20 ± 0.01 (2) a	6.08 ± 0.47 (64) a	3.41 ± 2.06 (33) a
Mn 0.1	0.18 ± 0.02 (1) a	6.23 ± 0.65 (39) a	9.74 ± 1.99 (59) b
Mn 1	0.28 ± 0.05 (2) a	4.18 ± 0.21 (28) b	9.33 ± 2.61 (55) b
Mn 100	0.54 ± 0.09 (5) b	0.67 ± 0.06 (7) c	4.11 ± 0.31 (41) a
Cu 100	1.19 ± 0.15 (14) c	1.12 ± 0.16 (13) c	0.75 ± 0.3 (9) a
K content:			
Control	0.09 ± 0.28 (1) a	0.01 ± 0.00 (0) a	15.2 ± 1.8 (96) a
Mn 0.1	0.03 ± 0.00 (0) a	0.01 ± 0.00 (0) a	14.4 ± 0.6 (96) a
Mn 1	0.01 ± 0.01 (0) a	0.02 ± 0.01 (0) a	16.5 ± 0.0 (93) a
Mn 100	0.00 ± 0.00 (0) a	0.04 ± 0.01 (0) a	15.2 ± 0.4 (92) a
Cu 100	12.1 ± 0.6 (51) b	1.84 ± 0.35 (8) b	4.03 ± 0.11 (17) c

Arithmetic means ± standard error. Numbers in brackets are percent values referring to the total amount of ions in the lichen thallus or released from the lichen thallus. The difference of the total of percent values to 100 % within a row is the percentage of ions released into the incubation medium. Within a column, separately for each element, means sharing a common letter do not differ significantly (Duncan's multiple range test, $P \leq 0.05$, $df = 10$). Controls run with deionized water.

Under natural conditions, the extracellular exchange sites in lichens are primarily occupied by Ca and Mg (BROWN & BECKETT 1984). Since the binding affinity of Mn to such sites is higher than that of Ca or Mg, extracellular Mn adsorption releases large amounts of Ca and Mg (HAUCK et al. 2002c, d; PAUL et al. 2003). This result parallels results obtained with other transition metals (BROWN & BECKETT 1984). Intracellular Ca concentrations were only slightly reduced (HAUCK et al. 2002d; PAUL et al. 2003), and intracellular Mg concentration never altered due to Mn uptake (HAUCK et al. 2002c, d; PAUL et al. 2003). The constant Mg content implies that Mn does not induce severe membrane damage (BRANQUINHO et al. 1997a). In contrast to other transition metals, such as Cu (BRANQUINHO et al. 1997a; CABRAL 2003), Pb (BRANQUINHO et al. 1997b)

or Ni (PUCKETT 1976), Mn does not induce K efflux (Table 12-3; HAUCK et al. 2002c, d; PAUL et al. 2003), which would indicate an increase of membrane permeability (GARTY et al. 1998). The lack of membrane damage may be one reason why the toxicity even of ambient Mn concentrations to lichens has been overlooked for so long, as membrane permeability was considered as a main criterion for heavy metal toxicity in early studies of this topic even more than today (BURTON et al. 1981). The significant K efflux induced by $MnSO_4$ *Peltigera canina* (GOYAL & SEAWARD 1982) was not confirmed in our studies with other species of cyanolichens so that there is probably no general trend for higher sensitivity of the membranes to Mn in cyanolichens than in chlorolichens (Chapter 9)

8. Interaction of calcium, magnesium, iron and silicon with manganese toxicity

There is experimental evidence from *H. physodes* that Ca, Mg, Fe and probably also Si reduced Mn uptake and alleviated Mn toxicity symptoms. Reduced extracellular adsorption and reduced intracellular uptake of Mn in the presence of Ca or Mg is a direct consequence of the competition of these ions with Mn for the extracellular exchange sites and perhaps also for transmembrane transport (HAUCK et al. 2002*c*). This is consistent with an alleviation of Mn-induced growth inhibition in *H. physodes* soredia exerted by Ca and Mg single or in combination (HAUCK et al. 2002d). Reduced extracellular adsorption and intracellular uptake of Mn due to the addition of Ca or Mg was also observed in the cyanolichens *Lobaria pulmonaria* and *Nephroma helveticum*, but remarkably not in *Leptogium saturninum*, where Ca and Mg had no effect on intracellular Mn uptake (Chapter 9). The mitigation of Mn toxicity in *H. physodes* soredia by addition of $FeCl_3$ suggests that Mn causes Fe deficiency in lichens, as known from cyanobacteria (CSATORDAY et al. 1984) or vascular plants (CLAIRMONT et al. 1986; EL-JAOUL & COX 1998). Partly compensation of Mn-induced chlorophyll degradation by $FeCl_3$ supports this view (HAUCK et al. 2003). Lower Mn/Si ratios in the cell walls of collapsed versus intact *Trebouxia* cells in soredia of *H. physodes* may indicate an alleviating effect of Si on Mn toxicity in lichens. Such effects of Si are known from vascular plants, but mechanisms are poorly understood (HORIGUCHI 1988; IWASAKI et al. 2002). Cases where correlations between the ratios of Mn to Ca, Mg or Fe and the cover of *H. physodes* and other epiphytic lichen species were found in the field indicate

together with the experimental results that the ratio of Mn to nutrient cations may sometimes be more significant than the Mn concentration itself. This agrees with results from organisms other than lichens (GOSS & CARVALHO 1992; ISSA et al. 1995; BLACKWELL et al. 1998). Si concentrations were not analyzed during our field work.

9. *Lecanora conizaeoides*, a manganese tolerant lichen

Chlorophyll concentrations and chlorophyll fluorescence of *L. conizaeoides*, cover of which was not found to be correlated with Mn concentrations in bark or stemflow, remained unaffected by treatment with excess concentrations of $MnCl_2$, albeit concentrations ten times higher than in *H. physodes* were applied (HAUCK et al. 2003). In contrast to $MnCl_2$, $CuCl_2$ reduced the concentrations of the chlorophylls *a* and *b* significantly in *L. conizaeoides* (HAUCK et al. 2003). The decisive cause of the high Mn resistance of *L. conizaeoides* is probably its ability of effective intracellular immobilization of excess Mn (PAUL et al. 2003). PAUL et al. (2003) found that most Mn was accumulated in the apothecia, viz. in polyphosphate bodies and in S-containing deposits, which might be glutathione. While the Mn concentrations in the cell lumina outside such deposits were much lower in *L. conizaeoides* than in *H. physodes* (PAUL et al. 2003), total intracellular Mn concentrations were not different between either species (HAUCK et al. 2002c). Extracellular adsorption of Mn is significantly lower in *L. conizaeoides* than in *H. physodes* (HAUCK et al. 2002c). Different sensitivities of the photobionts play probably no role for the different Mn sensitivities of *L. conizaeoides* and *H. physodes*. Material of both species sampled from the Harz Mountains constantly contained different ITS variants of *Trebouxia*, indeed, but the genotype found in *L. conizaeoides* is also known from some taxa thought to be much more sensitive to toxic chemicals than *L. conizaeoides*, such as *Pseudevernia cladonia* and *P. consocians* (Helms, Hauck & Friedl unpubl.).

10. Outlook: What we know and what we don't know about manganese toxicity in epiphytes

Though never considered as a relevant site factor for epiphytic lichens until recently, there is now multiple evidence from the field and from laboratory experiments that high Mn concentrations limit the abundance of many epiphytic lichen species including *H. physodes*, whereas *L. conizaeoides* is, so far, the only lichen species studied, which is tolerant to high concentrations of Mn. Relating laboratory results to field data is always problematic, especially in the present case, because little is known about the availability of ions from the substrate. Ongoing experimental work with soredia of *H. physodes* transplanted to *Picea abies* bark of known Mn content and exposed for two vegetation periods and one winter in a spruce forest of the Harz Mountains is planned to link the laboratory and field data. Preliminary results from this experiment support previous conclusions made in the papers of HAUCK et al. (2002c, d, 2003) and PAUL et al. (2003, 2004) that Mn is effective at inhibiting lichen growth in the field. The soredia on control bark with low Mn content grow well, whereas on bark with high Mn content the soredia have difficulties to establish. Simultaneously, the Mn-rich bark is colonized by *L. conizaeoides*. The Mn concentrations used in this experiment correspond to ambient concentrations found in *Picea abies* bark in the Harz Mountains (Paul & Hauck unpubl.).

While these results suggest a relevance of the naturally occurring variation of Mn concentrations in bark and stemflow for the performance of epiphytic lichens in coniferous forests of the northern hemisphere, some questions are still open. It is unclear, for instance, whether Mn is also a relevant site factor in deciduous forest, where pH values in the soil, bark and stemflow are usually higher and, thus, Mn is less soluble. Apart from lichens, it would be very interesting to include other epiphytic organisms in the studies of Mn toxicity. In this context it would be interesting, for example, to study whether the often poor epiphytic bryophyte vegetation of conifers is, among other factors as microclimate, also the result of high Mn concentrations. Furthermore, it is unknown whether the distribution of non-epiphytic lichens is controlled by the supply with Mn. Lower Mn concentrations extractable from gabbro rock with rich lichen vegetation than from gabbro with sparse lichen cover in Ireland (BOYLE et al. 1987) suggest a possible significance of ambient Mn levels for saxicolous lichens, as well, but

no studies have been carried out to investigate this special case or any possible interaction between the performance of rock-dwelling lichens and their Mn supply. In case of the rock communities investigated by BOYLE et al. (1987) the lichen-rich rocks were simultaneously characterized by higher supply with P and K, which could also be decisive for the differences in lichen vegetation.

Summary

The effect of Mn on the viability of the epiphytic lichens, with special reference to *Hypogymnia physodes*, was studied.

Soredia of the lichen *Hypogymnia physodes* cultivated on agar plates for 8 days exhibited decreasing growth rates along with increasing Mn concentrations above 3 mM. Ca and Mg alleviated Mn toxicity. The chlorophyll content of the soredia was reduced under the influence of Mn and was positively correlated with the rate of grown soredia. *Trebouxia* cells of the soredia grown with excess Mn were smaller than control cells, had reduced chloroplasts and were partly collapsed. Fungal hyphae were shortened and strongly swollen. Disintegrated cell walls occurred both in algal and fungal cells. Intracellularly, Mn was accumulated in polyphosphate granules both in algal and fungal cells. Mn uptake was correlated with significant loss of Na, Mg and Ca, particularly from the mycobiont.

Element distribution was studied in soredia of the epiphytic lichen *H. physodes* cultivated for up to 11 days on agar medium with 500 µM $MnCl_2$. Mn concentrations in cell walls, cell lumina and polyphosphate granules of the mycobiont were elevated after 4 days. In the photobiont of sucessfully grown soredia, Mn concentrations in cell lumina and polyphosphate granules were not altered even after 11 days. This suggests that the cell interior of the photobiont as the most sensitive part of the soredium can temporarily be protected against the uptake of toxic Mn concentrations by Mn adsorption at the fungal and algal cell walls as well as by intracellular Mn immobilization in the poly-phosphate granules of the mycobiont.

The ultrastructure of soredia of the foliose epiphytic lichen *H. physodes* cultivated on agar plates for 11 days with three different concentrations of $MnCl_2$ (7 µM [control], 500 µM, 7 mM) was compared by means of TEM.

Photobiont cells of Mn-exposed soredia had swollen and contracted thylakoids. The chloroplasts were reduced in size, and numerous lysosome-like vesicles occurred in cells with degenerated chloroplast. At 500 µM, autospores of *T. jamesii* were more severely damaged than vegetative cells. At 7 mM, autospore formation was strongly reduced. Concentric bodies and mesosome-like structures were nearly completely absent from mycobiont cells of Mn-treated soredia at both 500 µM and 7 mM. At 500

μM, the photobiont of not grown soredia was more severely damaged compared to grown soredia (defined as soredia where division of algal cells had taken place). At 7 mM, ultrastructural damage was observed in both grown and not grown soredia. This suggests that even soredia that survived the first days of cultivation are probably not capable of forming thalli on Mn-rich substrates.

Exposure of thallus pieces of *H. physodes* to 100 mM $MnCl_2$ led to rapid adsorption to extracellular cation exchange sites; saturation was reached within about 50 min. Intracellular uptake was constant in samples exposed to 100 mM $MnCl_2$ for 10 to 120 min and analyzed for their Mn concentration after one day of recovery in the growth chamber. About 15 times as much Mn was bound extracellularly than was taken up intracellularly. Both 50 mM $CaCl_2$ and 50 mM $MgCl_2$ applied together with 100 mM $MnCl_2$ significantly reduced Mn uptake intracellularly and binding in the extracellular exchange sites. $CaCl_2$ was more effective than $MgCl_2$ at reducing Mn uptake intracellularly; extracellularly $CaCl_2$ and $MgCl_2$ reduced Mn adsorption equally. *H. physodes* bound significantly more Mn from 1 - 100 mM $MnCl_2$ solutions on its extracellular exchange sites than *Lecanora conizaeoides*.

Thallus pieces of *H. physodes* and of the Mn-resistant *Lecanora conizaeoides* were incubated in 5 mM $MnCl_2$ for 1 h. Element concentrations and thallus structure were subsequently studied with SEM, TEM and X-ray microanalysis. Mn concentrations both in fungal and algal cell walls and cell lumina were much lower in *L. conizaeoides* than in *H. physodes*, because the former immobilized Mn in the thallus and in apothecia. *H. physodes* also immobilized some Mn in extracellular particles in the upper cortex and in intracellular polyphosphate granules in the lower cortex. However, extra- and intracellular Mn concentrations in *H. physodes* increased much more during incubation with Mn compared to *L. conizaeoides*. The highest Mn concentrations were found in the upper and the lower cortex (i.e. in the cell walls and in the interhyphal polysaccharide matrix). The photobiont of *H. physodes* took up considerably less Mn than the mycobiont; this suggests that the latter is capable of protecting the photobiont to a certain extent from Mn invasion. Mn uptake released much Ca and Mg from *H. physodes*, especially from cortical cell walls and polysaccharide matrices. In the medulla, Mn was incorporated in Ca oxalate crystals especially on the surface of young growing hyphae.

Concentrations of chlorophylls *a* and *b* decreased with increasing $MnCl_2$ supply in the epiphytic lichen *H. physodes*, but not in *L. conizaeoides*. $FeCl_3$ compensated for $MnCl_2$-

induced chlorophyll degradation in *H. physodes*. $MnCl_2$ did not affect the concentrations of ATP, ADP and AMP in *H. physodes*.

The cytoplasma membranes (at least of the mycobiont) in the epiphytic lichens *Alectoria sarmentosa* and *Bryoria fuscescens* were like in the previously studied *H. physodes* not damaged by $MnCl_2$, since intracellular concentrations of K and Mg were not significantly affected. $MnCl_2$ displaced significant amounts of Mg from extracellular exchange sites.

Mn uptake from $MnCl_2$ solution and chlorophyll fluorescence were studied in the epiphytic lichens *Leptogium saturninum, Lobaria pulmonaria and Nephroma helveticum*. Extracellular adsorption and intracellular uptake of Mn increased in the order *L. pulmonaria* < *N. helveticum* < *L. saturninum*. Mn increasingly reduced the effective quantum yield of photochemical energy conversion in photosystem 2 (Φ_2) in the same order. $CaCl_2$ and $MgCl_2$ alleviated the Mn-induced reduction of Φ_2.

Incubation with 10 mM $MnCl_2$ for 1 h decreased the Φ_2 under different light regimes in the lichens *H. physodes, L. saturninum* and *N. helveticum,* but not in *L. pulmonaria.* Among the bipartite species, Mn sensitivity increased in the order *H. physodes* < *N. helveticum* < *L. saturninum.* This equals the sequence heteromerous chlorolichen < heteromerous cyanolichen < homoiomerous cyanolichen. $MnCl_2$ reduced non-photochemical quenching of chlorophyll fluorescence in the bipartite cyanolichens and in *H. physodes.* Photochemical quenching was increased in *H. physodes,* but reduced in the bipartite cyanolichens. These results indicate that the bipartite cyanolichens *L. saturninum* and *N. helveticum* are even more sensitive to high Mn concentrations than the chlorolichen *H. physodes*. The high sensitivity of the bipartite cyanolichens can be attributed to a high sensitivity of the *Nostoc* photobiont, as sensitivity was highest in the gelatinous *L. saturninum,* which has a higher photobiont/mycobiont ratio than the less sensitive homoiomerous *N. helveticum.* The high Mn tolerance of *L. pulmonaria* is probably not due to its being a tripartite lichen, but might be caused by high tolerance of the green-algal primary photobiont *Dictyochloropsis,* which is, however, not experimentally proven. The high Mn tolerance of the highly SO_2-sensitive *L. pulmonaria* shows that different mechanisms are responsible for Mn and SO_2 toxicity in lichens.

Chlorophyll fluorescence microscopy was applied to assess the sensitivity to excess concentrations of Mn in *H. physodes*, *L. conizaeoides*, *L. saturninum*, *L. pulmonaria*

and *N. helveticum*. Different sensitivity of the PS2 towards Mn was found for cyano- and chloro-lichens. The fluorescence signal in *L. saturninum* was most strongly affected by excess Mn. Reduced intensity of the fluorescence signal was also visually perceptible in Mn treated thalli of the *N. helveticum*, but high variability was apparently responsible for lacking statistical significance. The chlorolichen *H. physodes* showed a significant reduction of the intensity of the fluorescence signal. The fluorescence signal was as insensitive to excess Mn in *L. pulmonaria* as in *L. conizaeoides*.

Decreasing abundance of epiphytic lichens with increasing Mn supply from the substrate or from stemflow was found in several coniferous forests of Europe (Germany) as well as western (Montana, British Columbia) and eastern North America (New York State). Experiments carried out with *H. physodes* and other species of chloro- and cyano-lichens suggests that these correlations are causal. High Mn concentrations, e.g., reduce chlorophyll concentrations, chlorophyll fluorescence and degrade the chloroplast in lichen photobionts. Excess Mn inhibits the growth of soredia of *H. physodes* and causes damage in the fine- and ultra-structure of the soredia. Adult lichen thalli remain structurally unaffected by Mn. Mn uptake does not result in membrane damage. Ca, Mg, Fe and perhaps also Si alleviate Mn toxicity symptoms in *H. physodes*. *L. conizaeoides* is not sensitive to Mn both in laboratory experiments and in the field.
The data suggest that high Mn concentrations are an important site factor for epiphytic lichens in coniferous forests that has been overlooked until recently.

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