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Uptake and toxicity of manganese in epiphytic cyanolichens

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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 bipartite 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.
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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., 2002b,c, 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 and Hauck, 2003). 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 and Wolterbeek, 1993; Levia and 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., 2001, 2002a) and experimental evidence (Hauck et al., 2002b, 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 and Gries, 2002).

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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 and 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 chlorolichens 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 *L. pulmonaria* resulted in the hypothesis that bipartite cyanolichens are more sensitive to Mn than tripartite lichens with cephalodia. To test these hypotheses, three species were selected that occur on conifers of northwestern Montana (Hauck and Spribille, 2002) and represent three different types of cyanolichens. *Leptogium saturninum* is an homoiomerous, gelatinous, bipartite cyanolichen, *N. helveticum* is an heteromerous, bipartite cyanolichen lichen, and *L. 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 and 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,b,c, 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 *L. saturninum* (Dickson) Nyl., *L. pulmonaria* (L.) Hoffm. and *N. 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 1 day at 80% relative humidity, a day temperature (for 13 h daily) of 13 °C during a photon flux of 30 $\mu mol\ m^{-2}\ s^{-1}$, 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 and 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 and 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 incuba-

tion 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 Section 2.1, for 2 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 2 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 $\lambda = 655$ 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 2). Statistical analyses were computed with SAS 6.04 software (SAS Institute Inc., Cary, North Carolina, USA). Michaelis-Menten regression lines for intra- and extra-cellular Mn uptake in Figs. 1–4 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 1). Mn uptake versus time was studied in *L. pulmonaria* and *N. helveticum* (Fig. 1, Table 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} dry weight. Intracellular uptake reached saturation within a few minutes in both species (Fig. 1). 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, d.f. = 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

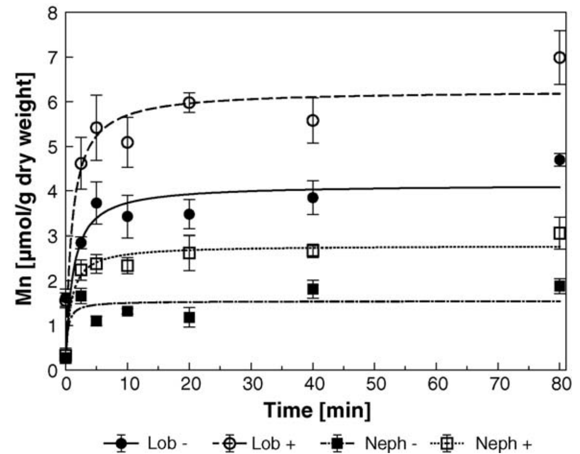

Fig. 1. Intracellular Mn uptake in *L. pulmonaria* and *N. helveticum* related to time from 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob−, Neph−) or after a subsequent recovery day in the growth chamber (Lob+, Neph+). Arithmetic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

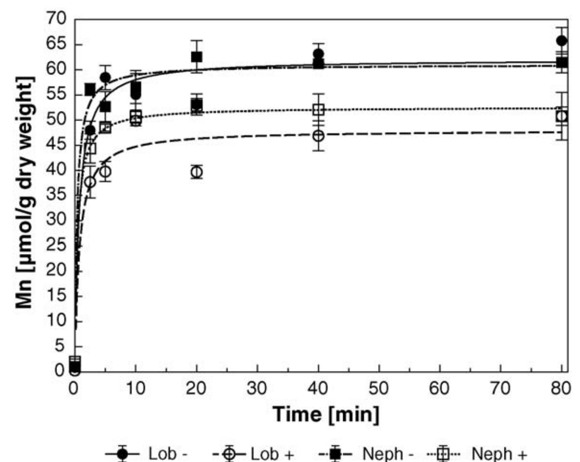

Fig. 2. Extracellular Mn adsorption in *L. pulmonaria* and *N. helveticum* related to time from 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob−, Neph−) or after a subsequent recovery day in the growth chamber (Lob+, Neph+). Arithmetic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

Table 1
Intracellular concentrations of Mn, Ca, Mg, K (in μmol g^{-1} dry weight) and molar ratios of Ca and Mg to Mn in *L. pulmonaria*, *N. helveticum* and *L. 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

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

Arithmetic mean ± S.E. Statistics: Duncan's multiple range test, $P \leq 0.05$, d.f. = 16. Within a row, means sharing a common letter do not differ significantly.
[a] Recovery: +, 24 h recovery after treatment; −, without recovery day.

high as in the cell interior (Fig. 2, Table 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 dry weight, and in the latter 8.6 ± 1.1 μmol g^{-1} Mn dry weight were removed from the extracellular cation exchange sites. In *L. saturninum*, 3.2 μmol g^{-1} Mn dry weight 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 1), while extracellularly bound Mn concentrations were not different (Table 3).

At the extracellular exchange sites, Mn uptake strongly reduced concentrations of Ca (Fig. 3) and Mg (Fig. 4). In all species, extracellular Ca and Mg content increased during the recovery day (Figs. 3 and 4; Table 3). Mn uptake had no significant effect on intracellular concentrations of K, Ca and Mg (Tables 1 and 2). Thus, intracellular Ca/Mn and Mg/Mn ratios decreased only because of increased Mn concentrations (Tables 1 and 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 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 3).

Table 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 *L. pulmonaria* and *N. 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; d.f. = 27; 1, 6, 19).

Table 3
Concentrations of extracellularly bound Mn, Ca, Mg, K (in μmol g^{-1} dry weight) and molar ratios of Ca and Mg to Mn in *L. pulmonaria*, *N. helveticum* and *L. 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

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

Arithmetic mean ± S.E. Statistics: Duncan's multiple range test, $P \leq 0.05$, d.f. = 16. Within a row, means sharing a common letter do not differ significantly.
[a] Recovery: +, 24 h recovery after treatment; −, without recovery day.

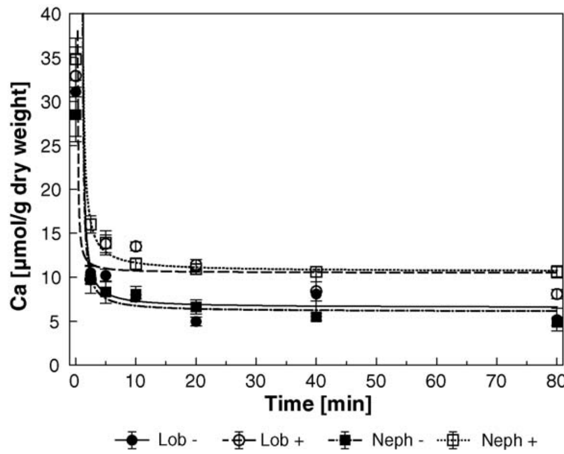

Fig. 3. Extracellularly bound Ca concentration in *L. pulmonaria* and *N. helveticum* related to time after incubation with 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob−, Neph−) or after a subsequent recovery day in the growth chamber (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. 5). In *N. helveticum*, 10 mM $MnCl_2$ reduced Φ_2 already after 2 days significantly (Fig. 6). In *L. saturninum*, 10 mM $MnCl_2$ reduced Φ_2 after 2 days, as did 5 mM $MnCl_2$ after two weeks (Fig. 7). Φ_2 of the samples where $MnCl_2$ was applied in combination with

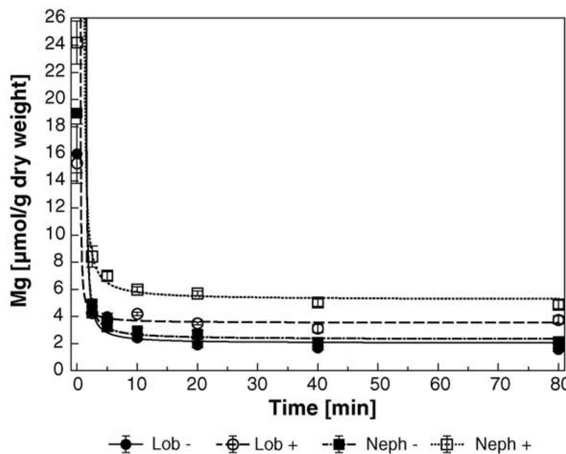

Fig. 4. Extracellularly bound Mg concentration in *L. pulmonaria* and *Nephroma helveticum* related to time after incubation with 5 mM $MnCl_2$ solution (Michaelis-Menten fits). Samples were analyzed immediately after the exposure to $MnCl_2$ (Lob−, Neph−) or after a subsequent recovery day in the growth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

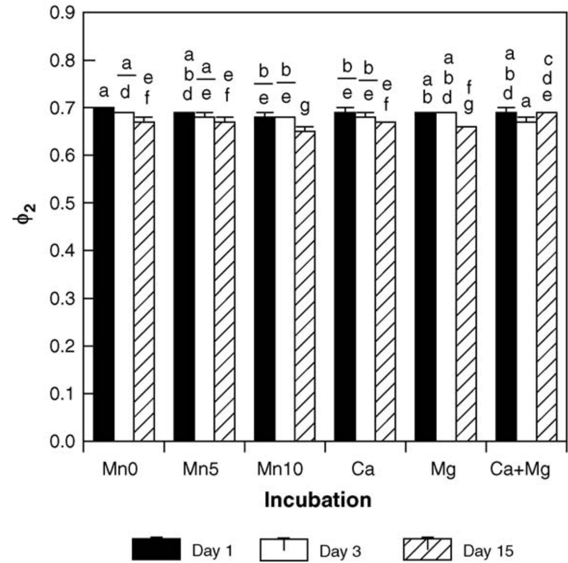

Fig. 5. Effective quantum yield of photochemical energy conversion in photosystem 2 (Φ_2) in *L. pulmonaria* before (day 1) and 2 days (day 3) or 2 weeks (day 15) subsequent to 40 min incubation with either deionized water (Mn0), 5 or 10 mM $MnCl_2$ (Mn5, Mn10), 5 mM $MnCl_2$ and 2.5 mM $CaCl_2$ (Ca), 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, $P \leq 0.05$, d.f. = 160). A line between two letters above a column includes all letters in the alphabet between these letters. Vertical bars indicate ± standard error.

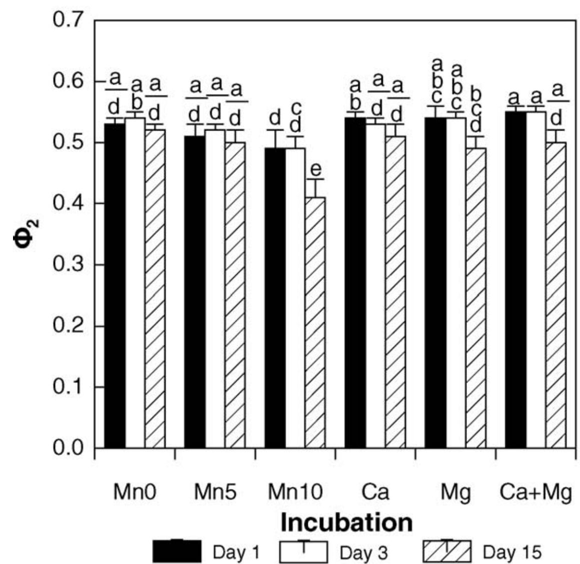

Fig. 6. Effective quantum yield of photochemical energy conversion in photosystem 2 (Φ_2) in *N. helveticum* before (day 1) and 2 days (day 3) or 2 weeks (day 15) subsequent to 40 min incubation with either deionized water (Mn0), 5 or 10 mM $MnCl_2$ (Mn5, Mn10), 5 mM $MnCl_2$ and 2.5 mM $CaCl_2$ (Ca), 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, $P \leq 0.05$, d.f. = 160). A line between two letters above a column includes all letters in the alphabet between these letters. Vertical bars indicate ± standard error.

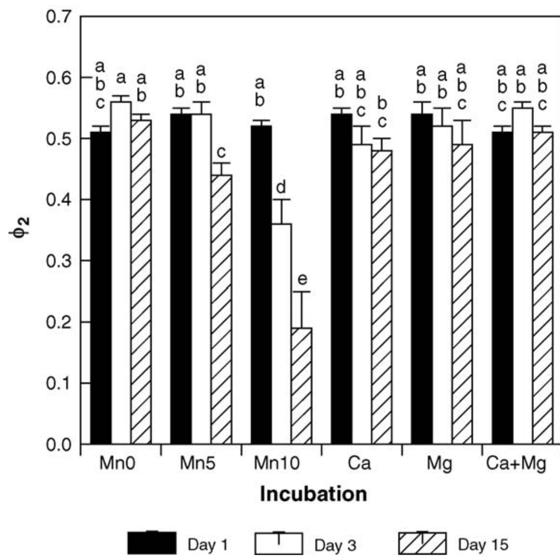

Fig. 7. Effective quantum yield of photochemical energy conversion in photosystem 2 (Φ_2) in *L. saturninum* before (day 1) and 2 days (day 3) or 2 weeks (day 15) subsequent to 40 min incubation with either deionized water (Mn0), 5 or 10 mM $MnCl_2$ (Mn5, Mn10), 5 mM $MnCl_2$ and 2.5 mM $CaCl_2$ (Ca), 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, $P \leq 0.05$, d.f. = 160). Vertical bars indicate ± standard error.

Ca and/or Mg did not significantly differ from Φ_2 of the controls.

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 *L. 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 and 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 and 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 *H. 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 and 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*, *N. helveticum*, *N. parile*, and *N. resupinatum* to conifer bark with the lowest Mn concentrations in the study of Hauck and Spribille (2002) from northwestern Montana was causal. This supports the hypothesis of Hauck and Spribille (2002) that the 'dripzone effect' of Goward and 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 and Arsenault (2000) and Hauck and 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 and 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 in cyanolichens 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., 2002b,c). Further, the Mn/Ca ratio is known to be signifi-

cant 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 and 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., 2002c). 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 and 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 and 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 and 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 and 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.

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