Cell-wall polysaccharides play an important role in decay resistance of *Sphagnum* and actively depressed decomposition in vitro

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Abstract *Sphagnum*-dominated peatlands head the list of ecosystems with the largest known reservoirs of organic carbon (C). The bulk of this C is stored in decomposition-resistant litter of one bryophyte genus: *Sphagnum*. Understanding how *Sphagnum* litter chemistry controls C mineralization is essential for understanding potential interactions between environmental changes and C mineralization in peatlands. We aimed to separate the effects of phenolics from structural polysaccharides on decay of *Sphagnum*. We measured aerobic microbial respiration of different moss litter types in a lab. We used chemical treatments to step-wise remove the chemical compounds thought to be important in decay-resistance in three taxonomically distant moss genera. We also focused on the effect of *Sphagnum*-specific cell-wall pectin-like polysaccharides (sphagnan) on C and N mineralization. Removing polymeric lignin-like phenolics had only negligible effects on C mineralization of *Sphagnum* litter, but increased mineralization of two other bryophyte genera, suggesting a minor role of these phenolics in decay resistance of *Sphagnum* but a major role of cell-wall polysaccharides. Carboxyl groups of pectin-like polysaccharides represented a C-source in non-*Sphagnum* litters but resisted decay in *Sphagnum*. Finally, isolated sphagnan did not serve as C-source but inhibited C and N mineralization instead, reminiscent of the effects reported for phenolics in other ecosystems. Our results emphasize the role of polysaccharides in resistance to, and active inhibition of, microbial mineralization in *Sphagnum*-dominated litter. As the polysaccharides displayed decay-inhibiting properties hitherto associated with phenolics (lignin, polyphenols), it raises the question if polysaccharide-dominated litter also shares similar environmental controls on decomposition, such as temperature or nutrient and water availability.

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Introduction

Sphagnum-dominated peatlands play an important
role in the global carbon (C) cycle. Peatlands in the
Northern hemisphere store between 270 and
370 Pg C (1 Pg = 10^{15} g) as peat (Turunen et al.
2002), amounting to 34–46% of the CO_2 currently
held in the atmosphere (IPCC 2007). In addition this
ecosystem sequesters about 12% of current human
emissions (Clymo et al. 1998). Understanding the
mechanisms responsible for the build up of the peat
deposits is of utmost importance to predict the risk of
destabilising this huge C store.

Up to 90 vol.% of the peat in *Sphagnum*-domi-
nated peatlands consists of litter of a single moss
genus: *Sphagnum* (Kuhry 1997; Turetsky 2003).
Sphagnum does not posses extraordinary high
primary production but, because of a slow rate of
decomposition, its litter accumulates as peat. The
slow litter decomposition in peatlands is generally
attributed to the synergic effects of environmental
factors, such as acidic and nutrient-poor soil condi-
tions above and anoxic conditions below the water
table, and the chemical composition of the litter,
particularly *Sphagnum* (Rydin and Jeglum 2006).
Although the latter is generally accepted to be
important (van Breemen 1995; Turetsky et al. 2008)
there is much debate about which compounds exactly
confer the high decay resistance of *Sphagnum* litter,
which resembles the resistance of woody tissues
(Moore and Basiliko 2006).

One train of thought emphasizes the importance of
phenolic compounds in *Sphagnum*. The cell-wall
surface of *Sphagnum* mosses is coated with amorphous
phenolic polymers, structurally and functionally sim-
ilar to lignin (phenylpropane-based), presumably
forming a physical barrier against decomposition
(Tsuneda et al. 2001). In turn, these lignin-like
phenolics decompose into soluble monomers such as
sphagnum acid (Rudolph and Samland 1985; Williams
et al. 1998). Together with other low-molecular-
weight soluble phenolics excreted by living cells
(Rasmussen et al. 1995), they are assumed to inhibit

the activity of extracellular enzymes, particularly
under anaerobic conditions (Verhoeven and Liefveld
1997; Freeman et al. 2001). Although there is correl-
ative evidence between the content of soluble
phenolics in *Sphagnum* litter and its aerobic
C-mineralization rate (Bragazza et al. 2006), antimi-
crobial properties of the phenolics are debatable (cf.,
Mellegård et al. 2009).

Another train of thought focuses on the role of
Sphagnum cell-wall polysaccharides. Resource parti-
tioning into metabolic and structural carbohydrates
was found to predict the rate of litter mineralization
(Turetsky et al. 2008) suggesting a passive mecha-
nism of resistance to decomposition. Moreover, there
are also indications of inhibitory, active mechanisms.
Cell-wall holocellulose of *Sphagnum* mosses contains
pectin-like rhamnogalacturonan I type polysaccha-
rides (Ballance et al. 2007) together with xylogluco-
mannan and cellulose (Painter and Sørensen 1978;
Painter 1983). Fragments of these pectin-like poly-
mers are slowly released into the environment as so
called sphagnan when the cell wall undergoes
autogenic acid hydrolysis (Painter 1991). Uronic
acids contained in the free or cell-wall bound
sphagnan may either directly or indirectly inhibit
microbial activity (Stalheim et al. 2009). Besides
lowering pH, the direct effect may involve interaction
between dissociated carboxyl groups of sphagnan and
the free amino groups of extracellular enzymes,
rendering the enzymes ineffective by forming a
polyelectrolyte complex. This process occurs in
solutions of low ionic strength between pH 2.0 and
4.8 (Ballance et al. 2008), i.e., conditions similar to
bog water and may lead not only to limitation of C
but also N mineralization, thus indirectly inhibiting
microbial growth. The significance of such electro-
static (ionic) reactions in more complex natural
environments has not yet been investigated. Earlier
experiments (Painter 1991; Børsheim et al. 2001)
suggesting irreversible condensation of amino groups
by carbonyl groups contained in sphagnan, were not
supported by later work (Ballance et al. 2008).

While soluble and polymeric phenolics are wide-
spread in plant tissues and their inhibitory effect on
microbial communities is generally known (Berg and
McClaugherty 2008 but cf. Mellegård et al. 2009),
the role of polysaccharides as inhibitors of decom-
position processes has not been studied. In this paper,
we attempted to differentiate the effects of lignin-like

polymeric phenolics from the effect of free and bound sphagnan on decay of *Sphagnum* litter. We hypothesize that free and bound sphagnan provide *Sphagnum* with unique properties among mosses in inhibition of microbial litter mineralization. To test our hypothesis, we designed four experiments in which we measured aerobic microbial respiration of different moss litter types in a lab environment. For the first three experiments we used chemical treatments to step-wise remove or de-activate the chemical compounds thought to be important in decay resistance in three taxonomically distant moss genera. This approach allowed us to systematically separate the influence of the different compounds. The first experiment tested the hypothesis that the low litter respiration rate of *Sphagnum* mosses in relation to other mosses is a result of its cell-wall polysaccharides, not of lignin-like phenolics. The second and third experiments focussed more on the relative importance of the mechanisms by which polysaccharides might affect C-mineralization in *Sphagnum* and non-*Sphagnum* litters, such as the role of carboxyl groups and environment in the form of pH. The fourth experiment took another approach. Here we applied isolated sphagnan to standardized *Sphagnum*-free plant litter to test the hypothesis that sphagnan inhibits microbial activity and thus C and N mineralization.

Materials and methods

Origin of litters

For experiments 1–3, we collected litter of four moss species, *Sphagnum magellanicum*, *Sphagnum majus*, *Leucobryum glaucum* and *Polytrichum strictum*. The moss litter was defined as recently dead (brown), usually 2 cm long shoot segments, which did not show signs of structural breakdown due to decomposition. *S. magellanicum*, *S. majus* and *P. strictum* were collected in a mountain ombrotrophic bog Rokytecká slať, Šumava National Park and Biosphere Reserve, Czech Republic (49°1′N, 13°25′E). *S. magellanicum* dominated in drier habitats such as lawns and low hummocks while *S. majus* occupied exclusively wet carpets. *P. strictum* dominated the highest hummocks, which were already too dry for any *Sphagnum* species. *Leucobryum glaucum* formed

typical cushions in the understory of nutrient-poor Scotch pine forest near Třeboň, Třeboňsko Protected Landscape Area and Biosphere Reserve, Czech Republic (48°59′N, 14°50′E). In the laboratory, we removed remaining living and decomposing tissues and other coarse plant remnants from the litter and washed all the litter with distilled water for about 15 min to remove fine debris. The litter was dried at 25°C and relative air humidity of 40%. Before it was fully dry, it was briefly homogenized using a kitchen mixer to obtain particles <5 mm long. This material, referred to as fresh litter, was poor in soluble phenolics (<1.0 mg of tannic acid standard per g of dry mass), as estimated using the Folin-Ciocalteu assay (Bärlocher and Graça 2005).

For experiment 4, recently senesced (brown) leaves of *Eriophorum angustifolium* were collected from Clara Bog, Ireland (53°51′N, 6°27′W). We choose this litter type for its common occurrence in peatlands and its relatively high decay rate (Limpens and Berendse 2003), likely ensuring measurable effect sizes. The leaves were dried at 70°C and ground to approximately 2-mm fragments. C:N of 48 indicated nutrient-poor litter quality.

Microbial inoculum

We inoculated litters with a suspension of a natural microbial community. We made a leachate from *S. magellanicum* litter collected above the water table from a bog.

For experiments 1–3, 1 kg of fresh litter was shaken in 3 l of de-ionized water for 1 h. The slurry was filtered through a fine polyamide mesh and coarse filter paper. Based on pre-experimental tests we added mineral nutrients to avoid nutrient limitation of microbial C mineralization rate (concentrations in mg l^{-1} are parenthesized): NH_4Cl (1000), K_2HPO_4 (1000), $MgSO_4 \cdot 7H_2O$ (200), $CaCl_2 \cdot 2H_2O$ (20), $FeCl_3 \cdot 6H_2O$ (10), $MnSO_4 \cdot H_2O$ (4), $CuSO_4 \cdot 5H_2O$ (1). For experiment 2, we used also doubled concentrations of salts of multivalent cations Mg^{2+}, Ca^{2+}, Fe^{3+}, and Mn^{2+}. The latter was done to avoid potential limitation of the decomposer community by essential metallic cations complexed preferably on carboxyl groups of the chlorite-treated then carboxyl-reduced litter (Thomas and Pearce 2004). The initial ionic strength of the inoculum was 31–35 mM, which is still favourable for sphagnan-protein complexation (Ballance et al. 2008).

For experiment 4, the litter of *S. magellanicum* was inserted into tubes (each 5 g fresh-weight) together with 30 ml of artificial rainwater (Garrels and Christ 1965) and 20 glass beads to facilitate dislodging of micro-organisms from the fibrous *Sphagnum* peat (Hopkins et al. 1991). The tubes were shaken at 5°C for 2 h and centrifuged at $500 \times g_n$ for 2 min and the supernatant collected. This procedure was repeated two times after re-suspending the residue with fresh rain water. To concentrate the micro-organisms collected with the water (c. 1120 ml in total), approximately half of the water was drained overnight using submerged Teflon-coated Rhizon soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands) at 5°C.

Experiment 1

To separate the effects of cell-wall polysaccharides and phenolics on microbial respiration, litters of four moss species: *S. magellanicum*, *S. majus*, *L. glaucum* and *P. strictum* were subjected to three different chemical pre-treatments to selectively remove a number of compounds. This resulted in three litter forms: (i) *fresh*, i.e., untreated dried litter, (ii) *chlorite-treated* and (iii) *borohydride-treated* which was incubated for 64 days, during which respiration was followed. In total there were 4 species × 3 treatments × 3 replicates = 36 samples. The pH was set to 3.5 in all samples using HCl.

Chlorite treatment involved removing the majority of aromatic polymers bound in cell walls. To this end fresh litter was boiled with acetone:methanol (2:1 v/v) for 6 times 5 min and bleached with sodium chlorite (Ballance et al. 2007). We thus obtained papery-white material which is assumed to be free of lignin-like phenolic polymers. This and subsequent treatments required careful manipulation with *L. glaucum* and particularly *P. strictum* because the bleached leaves became very fragile; *Sphagnum* structure was not noticeably affected. The C:N of fresh litter was about 65–90, and increased to about 110–270 after the chlorite treatment (lower values: *S. magellanicum* and *P. strictum*, higher values: *S. majus* and *L. glaucum*). We assume that adding 2.05 mg of NH_4^+–N per tube minimized potential effects of the initial litter N content (0.11–0.25 mg of N per tube) on C mineralization.

The chlorite treatment is commonly used for holocellulose quantification in plant tissues. It removes most of the lignin and other phenolics, as analyzed by optical spectrometry and confirmed by pyrolysis mass spectrometry (Morrison and Mulder 1995), and has a low impact on carbohydrate composition (Reeves 1993).

For the *borohydride treatment*, chlorite-bleached litter was further treated with sodium borohydride in order to reduce any carbonyl groups according to the standard procedure described in Ballance et al. (2007). The final borohydride-treated litter is assumed to be free of carbonyl groups. The litter C:N increased to 366–825 after borohydride treatment.

About 70.0 mg (oven-dry weight) of fresh or treated litter was inserted in 12-ml Exetainer glass tubes with screw septum cap. Samples were inoculated with 6 ml of microbial inocula and tubes were placed into an incubator at 15°C and relative humidity (RH) of >95% to minimize water evaporation from the uncapped tubes. 12 h prior to respiration measurements, the uncapped tubes were thoroughly vortexed, closed with a septum cap, and placed in the incubator horizontally to enhance gas exchange between the suspension and air. After the incubation period of 12 h, the capped tubes were vortexed and a 200-μl air sample was taken with a gas-tight syringe to analyze the CO_2 concentration on an Agilent 6850 gas chromatograph (Agilent Technologies, USA). During the incubation period 11–14 samplings were made; the CO_2 production was measured every second day in the beginning up to about once per 12 days at the end. Samples were vortexed at least every 3rd day to avoid anoxic conditions.

Measured CO_2 concentrations were converted to CO_2 production per initial C content per hour and these values were integrated in cumulative C loss for the whole 64-day period of the experiment. After the last measurement (64 days), the samples were lyophilized and weighted to obtain actual C loss. As the starting CO_2 concentration in the tubes was not measured directly after capping, this cumulative C loss is an overestimation of the actual C loss and needed to be proportionally adjusted based on the actual C loss (using Goal Seek function in MS Excel). Changes in relative C content of the samples during incubation were neglected in the calculations.

Experiment 2

To estimate the effect of cell-wall carboxyls on microbial respiration, the litters of two moss species, *S. magellanicum* and *L. glaucum*, were subjected to two chemical pre-treatments: (i) *chlorite-treatment* and (ii) *carboxyl-reduction,* amended with two concentrations of divalent metal ions (standard and doubled) in the inoculum, and incubated for 69 days during which respiration was followed. In total there were 2 species × 4 treatments × 3 replicates = 24 samples. pH was set to 3.5 in all samples using HCl.

Doubling the concentration of divalent cations did not affect C mineralization (Table 2), suggesting that the microbial community was not limited by availability of essential divalent cations and that the addition of cations (ionic strength of 4 mM) did not affect potential electrostatic interactions between litter carboxyls and microbial enzymes.

For details on chlorite treatment see experiment 1. *Carboxyl-reduction* involved the following procedure. We adapted the method based on the reduction of the carbodiimide-activated carboxyls (Stenutz et al. 2004). 1.2 g of chlorite-treated litter were suspended in 250 ml of water in a 2-l bottle and 50 ml of 0.2 M 2-(4-Morpholino-) ethanesulfonic acid, pH 4.75, were added, followed by 12 g of new N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (carbodiimide). After 4 h of stirring at room temperature, 250 ml of 2 M TRIS pH 9 were added and followed by a few drops of octanol to prevent foaming. After the sample had cooled in an ice bath, 250 ml of 17.5 g of $NaBH_4$ in 0.05 M NaOH was added and the suspension was kept overnight at 4°C and then slowly neutralized to pH 6 by addition of glacial acetic acid. Litter was collected by vacuum filtration, washed in 0.5 M NaCl and then in 20 mM HCl to convert the carboxyls into their H^+ form, washed in three changes of a large volume (8 l) of distilled water and once with acetone (500 ml). Finally, the litter was dried at room temperature and ready for incubation which followed the same procedure as for experiment 1.

To test the effectiveness of our carboxyl-reduction treatment, we determined the uronic acid (UA) content before and after carboxyl reduction using the assay of Ahmed and Labavitch (1978). Samples were mill-ground before analysis. The original contents of UA were 27.5 and 21.9% in *S. magellanicum*

and *L. glaucum*, respectively. It was reduced about 40% in *S. magellanicum* and 70% in *L. glaucum*. The C:N of 66 (*S. magellanicum*) and 98 (*L. glaucum*) in carboxyl-reduced litter suggests that some carboxyls were successfully activated by carbodiimide but have not been reduced by borohydride, as indicated by the lower C:N and smaller carboxyl reduction in *S. magellanicum*.

Experiment 3

To evaluate the effect of pH on microbial respiration of cell-wall polysaccharides, *chlorite-treated* litter of two moss species, *S. majus* and *L. glaucum*, was incubated at three levels of pH (3.5, 5.0 and 6.5) for 68 days during which respiration was followed. In total there were 2 species × 3 treatments × 3 replicates = 18 samples. The pH of the suspension was adjusted to 3.5, 5.0 or 6.5 by addition of either HCl or NaOH 6 h after inoculation and readjusted every 3–10 days. After each pH adjustment, a minimum calculated amount of NaCl was added to the samples to ensure equal ionic strength of the solution among all samples (up to 15 mM of Na^+). About 50% of carboxyls are dissociated at pH 3.5 and about 97% at pH 5.0.

For details on the chlorite treatment or respiration measurements, see experiment 1.

Experiment 4

To elucidate the effect of free soluble sphagnan on microbial respiration, *Eriophorum angustifolium* litter was amended with two polysaccharides: sphagnan (prepared from chlorite-treated litter of *S. papillosum* after Ballance et al. 2008) and polygalacturonic acid (PGA, Sigma P-3850, 79% of galacturonic acid) in five concentrations (0, 5, 10, 20, and 50 mg g^{-1} l; dry weight equivalent) and incubated for 30 days. Sphagnan and PGA were added in the same concentrations, as both compounds have a similar C-content per monomer. In total there were: 10 treatments × 3 replicates = 30 samples. After the incubation cumulative C mineralization and changes in dissolved inorganic (DIN) and organic nitrogen (DON) fractions were measured.

The ground litter of *E. angustifolium* was mixed with quartz sand (Merck) and inoculum in a weight-ratio of 1:3:5. This ratio was found to be optimal for

microbial respiration in a previously conducted pilot experiment. The substrate was then covered and pre-incubated at 20°C for 1 week, during which the microbial community could build up and stabilize (Kraus et al. 2004). Hereafter the substrate was mixed thoroughly and portions of 15 g were divided over 35 plastic containers (4.5 cm in diameter, height of 5.0 cm). Five containers with substrate were kept apart for chemical analyses. To compensate for the N lost during the pre-incubation period, 0.23 mg of N was added as NH_4NO_3 to each of 30 containers. The added amount was based on the loss of mineral N during the pre-incubation period of the pilot experiment. After this, freshly prepared solutions of sphagnan or PGA (pH 4.0, adjusted with 0.1 M NaOH) were mixed through the substrates, and the whole containers were placed in 0.5 l airtight mason jars and fitted with a septum-covered outlet. A thin layer of de-ionized water was added to the jars to keep RH constant. The jars were placed in an incubator under 20°C for 30 days.

It was not possible to completely dissolve PGA to prepare a 50 mg g^{-1} solution so this treatment was omitted from the experiment. The pH (KCl) at the end of the experiment ranged between 3.7 for the lowest concentration and 4.3 or 4.2 for the highest concentrations of sphagnan or PGA.

Chemical analyses

The initial C and N content of all the moss species and their litter forms (experiment 1–3) was determined by an Elemental analyzer (ThermoQuest Italia, Milan, Italy) in two replicates (coefficient of variation <8%). To be aware of possible N-limitation of C mineralization in experiments 2 and 3, we centrifuged the tubes at $5000 \times g_n$ for 10 min at the end of incubation and used flow injection analysis to determine the concentration of available NH_4^+ and NO_3^- in the supernatant. The concentration of NO_3^- was negligible in all samples so NH_4^+ represented the available DIN. No losses of DIN are expected through denitrification due to aerobic experimental conditions.

In experiment 4, we measured cumulative C mineralization as well as the changes in the DIN and DON. Soil samples were taken after pre-incubation before adding extra N (five containers), as well as at the end of the experiment (all containers). Prior

to sampling, the substrate was mixed thoroughly in the containers and sub-samples were taken to determine water and ash contents, C:N, total dissolved nitrogen (TDN), nitrate nitrogen (N–NO_3) and ammonium nitrogen (N–NH_4). Water content was determined after drying at 105°C and ash content by ignition at 550°C. The substrate was ground in a ball-mill and its C content was determined on an elemental analyzer (Fisons Instruments EA 1108, Milan Italy). To determine the available N fractions, 8 g of fresh substrate was extracted with 20 ml 1 M KCl by shaking for 2 h. N–NO_3, and N–NH_4 were measured colorimetrically using a continuous flow analyser (SKALAR SAN plus system, the Netherlands). TDN was measured as N–NH_4 after Kjeldahl digestion.

We express C loss per initial C content in %. The amount of extra C added to the substrate as sphagnan or PGA was included in these calculations. DIN represents the sum of N–NO_3 and N–NH_4 contents, DON = TDN − DIN.

Statistical analyses

Factorial general linear models (GLM) ANOVA were used to test effects of factors (species, treatment) on cumulative respiration at the end of incubation periods (experiments 2–3). Repeated measures (RM) GLM ANOVA was used to test factor effects on the course of respiration rates during incubation periods for all experiments. We employed nested design in case of experiment 1 to test the effect of groups (*Sphagnum* and non-*Sphagnum*), which both include two different species. Differences between means within factors were determined with Tukey's honestly significant difference (HSD).

In experiment 4, the highest sphagnan concentration (50 mg g^{-1} in which PGA did not dissolve) was not included in the RM ANOVA to keep the design balanced. The effect of adding the highest concentration of sphagnan was tested against the lowest concentration (no sphagnan added) using a two-tailed Student's *t*-test. The effect of C-mineralization rate on TDN and DIN was tested with an ANCOVA with substance as factor and concentration and C-mineralization as co-variables. C-mineralization rate had no effect on either TDN, DIN or DIN:DON ratio.

We used STATISTICA v. 8.0 software package. Statements given in Result section represent

statistically significant differences ($\alpha = 0.05$) unless otherwise indicated.

Results

Experiment 1: mineralization of fresh, chlorite-treated and borohydride-treated litter

Cumulative C mineralization (Fig. 1a) of fresh litter was similar in all species, ranging between 12 and 15% of the initial C concentration. Removing phenolics by chlorite treatment more than doubled C mineralization of non-*Sphagnum* species, whereas C mineralization of *Sphagnum* was hardly affected at all, leading to a strong litter × group interaction (Tab. 1). The above illustrates a key role for phenolic compounds as drivers of C-mineralization in *Leucobryum* and *Polytrichum* litter, but only a minor role in aerobic C mineralization of *Sphagnum*. Reduction of carbonyl groups after the borohydride treatment affected only litter of *S. magellanicum*, in which the C mineralization increased by 4% (Fig. 1a).

The C mineralization rate of fresh litters (Fig. 2a) decreased steadily after peaking at about 225 μg g^{-1} h^{-1} between 4th and 7th day, reaching a rate of 50 μg g^{-1} h^{-1} at the end of the incubation period for all species. In contrast, C mineralization rate of chlorite- and borohydride-treated litters, (Fig. 2b–c) reached a roughly constant rate of about 100 μg g^{-1} h^{-1} after 10 days in both *Sphagnum* species, but continued to increase in *P. strictum* and *L. glaucum* to 350 μg g^{-1} h^{-1} at the end of the experiment, leading to significant interactions of all factors with TIME (Table 1).

Experiment 2: mineralization of carboxyl-reduced litter

Cumulative C mineralization (Fig. 1b) of chlorite-treated litter of *S. magellanicum* was about 3.4 times lower than that of *L. glaucum*, and showed contrasting responses to carboxyl reduction between species (Table 2). While cumulative C mineralization increased by 4% in *S. magellanicum* it showed a similar decrease in *L. glaucum*, indicating that carboxyls represented a C source in the latter species.

The C mineralization rate of the carboxyl-reduced litter of *S. magellanicum* was far slower than in the
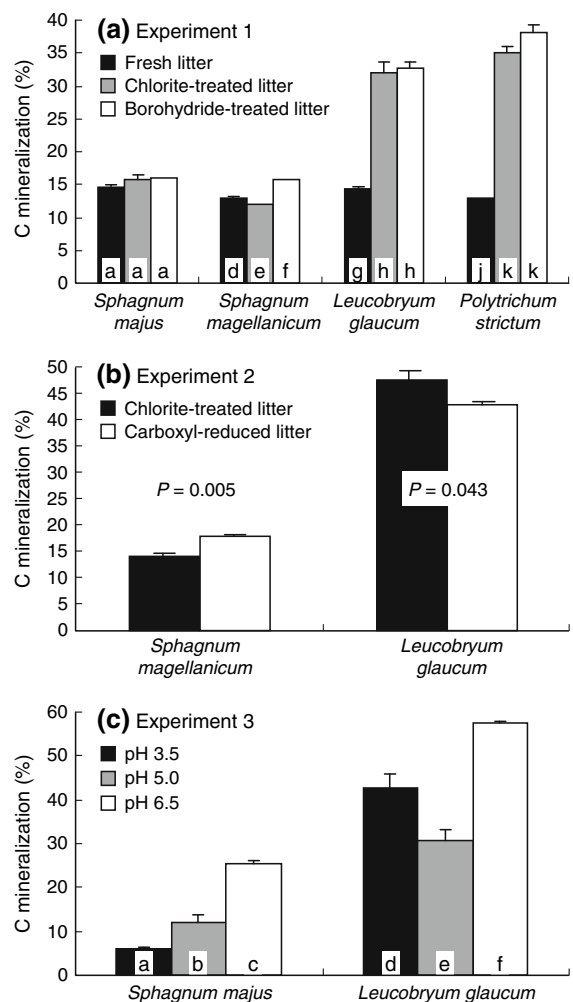

Fig. 1 Cumulative C mineralization (means + SE, $n = 3$) in modified moss litters after 64–69 days of incubation. The chlorite-treated litter is assumed to be free of phenolics, represented particularly by lignin-like polymers. The borohydride-treated litter is, in addition, free of carbonyls. The carboxyl content of carboxyl-reduced litter was reduced about 40% in *S. magellanicum* and 70% in *L. glaucum* in comparison with the chlorite-treated control. Different letters indicate significant differences between litter forms within species ($P < 0.05$; one-way ANOVA, Tukey's HSD test), P values refers to Student's *t*-test

original chlorite-treated litter during the initial 4 days (Fig. 3a). While the rate of C mineralization remained more or less stable during the 69-day period in *S. magellanicum*, it started to increase after about 30 days in *L. glaucum*. As a result the interactions of the repeated-measures factor TIME are statistically significant (Table 2).
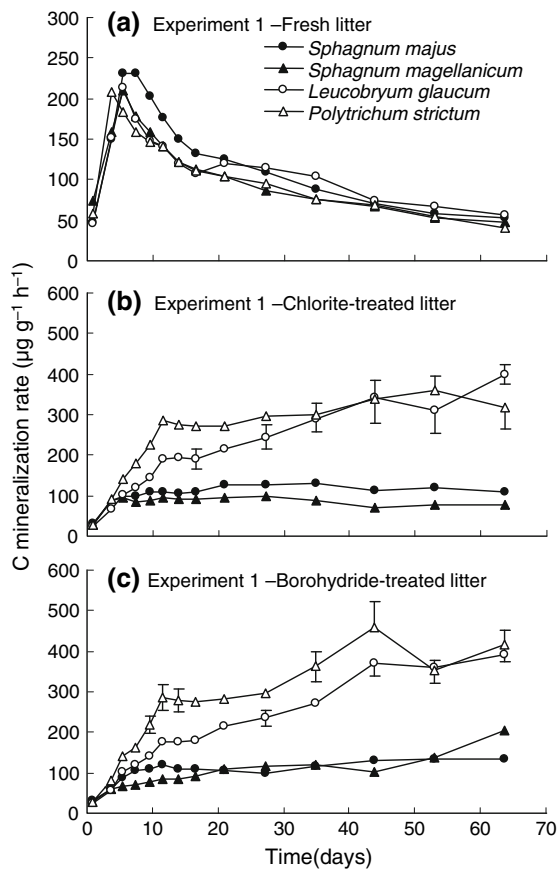
Fig. 2 The course of C mineralization rates in modified moss litters. The chlorite-treated litter is assumed to be free of particularly polymeric phenolics and the borohydride-treated litter, in addition, free of carbonyls. Means ± SE ($n = 3$); for readability, only relatively large SE values are shown. Note the different y-axis scales

Experiment 3: pH effect on C mineralization

The response of cumulative C mineralization to pH differed between species. It increased with increasing pH in chlorite-treated litter of *S. majus*. In *L. glaucum*, however, C mineralization was lowest at an intermediate pH of 5.0 and highest under the highest pH level of 6.5 (Figs. 1c and 3b). As a result the species × pH interaction was statistically significant (Table 3), indicating that acidity alone was not the only factor suppressing microbial activity with decreasing pH. The rate of C mineralization of *L. glaucum* at pH of 6.5 decreased in the last phase of the experiment (Fig. 3b), probably because of the exhausted content of available NH_4^+ that fell below

0.7 mM. In the other samples, NH_4^+ ranged between 5.9 and 12.5 mM (data not shown).

Experiment 4: effects of sphagnan and PGA addition on litter mineralization

Carbon mineralization responded differently to adding sphagnan and PGA, and was lowest for the sphagnan treatment (Fig. 4a, Table 4). Carbon mineralization remained unaffected by sphagnan addition up to 20 mg g^{-1}, but it was depressed by adding 50 mg g^{-1}. In contrast, C mineralization increased by adding 10 and 20 mg g^{-1} of PGA. The increase was such that it could not be explained by mineralisation of the added substrate alone, thus indicating increased decomposition of the plant litter, and a priming effect of PGA. The availability of nitrogen, expressed as total dissolved N (TDN) decreased from the initial 51 μg N g^{-1} to below 30 μg N g^{-1} at the end of the experiment for all treatments (Fig. 4c, Table 4). Thus, N uptake apparently exceeded mineralization indicating N co-limitation of the decomposer community. At the end of the experiment the TDN was positively related with sphagnan addition, while only the maximum PGA addition of 20 mg g^{-1} was followed by TDN increase (Fig. 4c). The concentration of dissolved inorganic N (DIN) decreased sharply from 23 μg N g^{-1} to below 3 μg g^{-1} during the experiment for both treatments (Fig. 4b), especially the concentration of nitrate which reached values below the detection limit (0.01 μg g^{-1}) at the end of the experiment. Despite this strong decline, there was still a treatment effect at the end of the experiment (Fig. 4b, Table 4). DIN concentration was lowest for the sphagnan treatment, indicating either a faster microbial uptake of DIN and/or lower N mineralization rate than in the PGA treatment. The addition of sphagnan and PGA had contrasting effects on the DIN concentrations: the more sphagnan added, the lower the DIN concentration, whereas the higher PGA addition, the higher the DIN concentration. In contrast with DIN, the concentration of dissolved organic N (DON) increased during the experiment in both treatments, particularly in sphagnan treatment (data not shown). As a result, the ratio between DIN and DON decreased from 0.8 to 0.2 and 0.1 for sphagnan and PGA, respectively, over the experimental period. At the end of the experiment, the ratio between DIN and DON differed between the

Table 1 Experiment 1: the effects of moss group (*Sphagnum* and non-*Sphagnum* mosses), species (*Sphagnum magellanicum* and *S. majus*; *Leucobryum glaucum* and *Polytrichum strictum*), litter form (fresh, chlorite-treated, and borohydride-treated) on cumulative respiration at the end of the incubation period (nested ANOVA) and on the course of respiration rates during the incubation period (nested repeated measures ANOVA)

Source	Cumulative respiration			Respiration rates		
	F	d.f.	P	F	d.f.	P
Group	237.5	1	<0.0001	134.2	1	<0.0001
Species (group)	7.3	2	0.0028	13.9	2	0.0001
Litter	159.1	2	<0.0001	41.5	2	<0.0001
Litter × group	127.2	2	<0.0001	96.4	2	<0.0001
TIME [repeated measure factor]				10.4	13	<0.0001
TIME × group				39.7	13	<0.0001
TIME × species(group)				3.7	26	<0.0001
TIME × litter				89.4	26	<0.0001
TIME × litter × group				16.0	26	<0.0001

Table 2 Experiment 2: the effects of species (*Sphagnum magellanicum* and *Leucobryum glaucum*), litter form (chlorite-treated and carboxyl-reduced) and cations (normal and doubled concentration of divalent cations) on cumulative respiration at the end of the incubation period (ANOVA) and on the course of respiration rates during the incubation period (repeated measures ANOVA)

Source	Cumulative respiration			Respiration rates		
	F	d.f.	P	F	d.f.	P
Species	2105.2	1	<0.0001	1298.0	1	<0.0001
Litter	0.5	1	0.4855	28.4	1	0.0001
Cations	0.0	1	0.8706	0.4	1	0.5616
Species × litter	55.4	1	<0.0001	33.4	1	<0.0001
Species × cations	0.2	1	0.6841	0.1	1	0.7531
Litter × cations	0.0	1	0.9880	0.1	1	0.7238
Species × litter × cations	0.4	1	0.5477	0.3	1	0.5934
TIME [repeated measures factor]				1030.4	11	<0.0001
TIME × species				663.1	11	<0.0001
TIME × litter				22.2	11	<0.0001
TIME × cations				0.2	11	0.9976
TIME × species × litter				16.7	11	<0.0001
TIME × species × cations				0.5	11	0.9203
TIME × litter × cations				0.1	11	0.9994
TIME × species × litter × cations				0.2	11	0.9991

treatments, with the lowest DIN:DON ratio for the sphagnan treatment and the highest DIN:DON ratio for the PGA treatment (Fig. 4d, Table 4). Increasing additions of sphagnan and PGA seemed to have contrasting effects; sphagnan addition decreased DIN:DON ratio but PGA addition resulted in its increase.

Discussion

We showed that polymeric lignin-like phenolics play a minor role in maintaining the low aerobic C-mineralization rate of *Sphagnum* litter in the short term (10 weeks), but do play a role in other moss genera. We cannot exclude, however, that *Sphagnum*
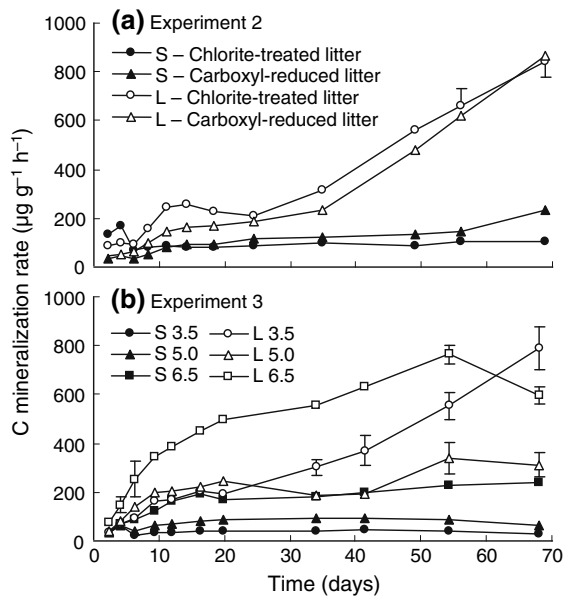
Fig. 3 The course of C mineralization rates in modified moss litters. *S Sphagnum magellanicum*, *L Leucobryum glaucum*; 3.5, 5.0 and 6.5 denote incubation pH. The chlorite-treated litter is assumed to be free of particularly polymeric phenolics. The carboxyl content of carboxyl-reduced litter was reduced about 40% in *S. magellanicum* and 70% in *L. glaucum* in comparison with the chlorite-treated control. Means ± SE ($n = 3$); for readability, only relatively large SE values are shown

phenolics in general, may indirectly limit aerobic C mineralization in bogs via nutrient limitation. That is, soluble phenolics, either those excreted by living *Sphagnum* or the lignin-like polymers that decompose into monomers, participate in formation of humic substances. This process is accompanied by

organic N and potentially P sequestration (Paludan and Jensen 1995) that may further intensify nutrient limitation already induced by the ombrotrophic character of the bog habitat. In other moss genera, such as *Leucobryum* and *Polytrichum*, phenolic polymers seem to form essential physical protection of polysaccharides against mineralization. For *Sphagnum* this mechanism seems to play a minor role towards the later phases of incubation (Fig. 2).

In accordance with our hypothesis, experiment 1 illustrated the importance of cell-wall polysaccharides in explaining the overall low C mineralization rate of *Sphagnum* litter relative to other moss litters (Lang et al. 2009). Whether differences in polysaccharide concentration also explain inter-specific differences in decay rate within *Sphagnum*, remains to be seen, although recent work (Turetsky et al. 2008) indicates that the ratio between easily and poorly degradable polysaccharide fractions is a good predictor for decay rates of *Sphagnum* species.

The negligible effect of carbonyl groups on C mineralization is consistent with recent works of Ballance et al. (2007, 2008) and Kristiansen et al. (2009) who did not find any evidence for abnormally large numbers of reactive carbonyls in *Sphagnum* cell-wall polysaccharides. Thus, the mechanisms, by which polysaccharides impede litter respiration, remain elusive. Interestingly, the proposed mechanisms so far show many similarities with those reported for phenolic compounds (Berg and McClaugherty 2008): sphagnan seems to be relatively stable in the environment (Painter 1991) and actively interferes with decomposers either directly or

Table 3 Experiment 3: the effects of species (*Sphagnum majus* and *Leucobryum glaucum*) and pH (3.5, 5.0 and 6.5) on cumulative respiration at the end of the incubation period

(ANOVA) and on the course of respiration rates during the incubation period (repeated measures ANOVA)

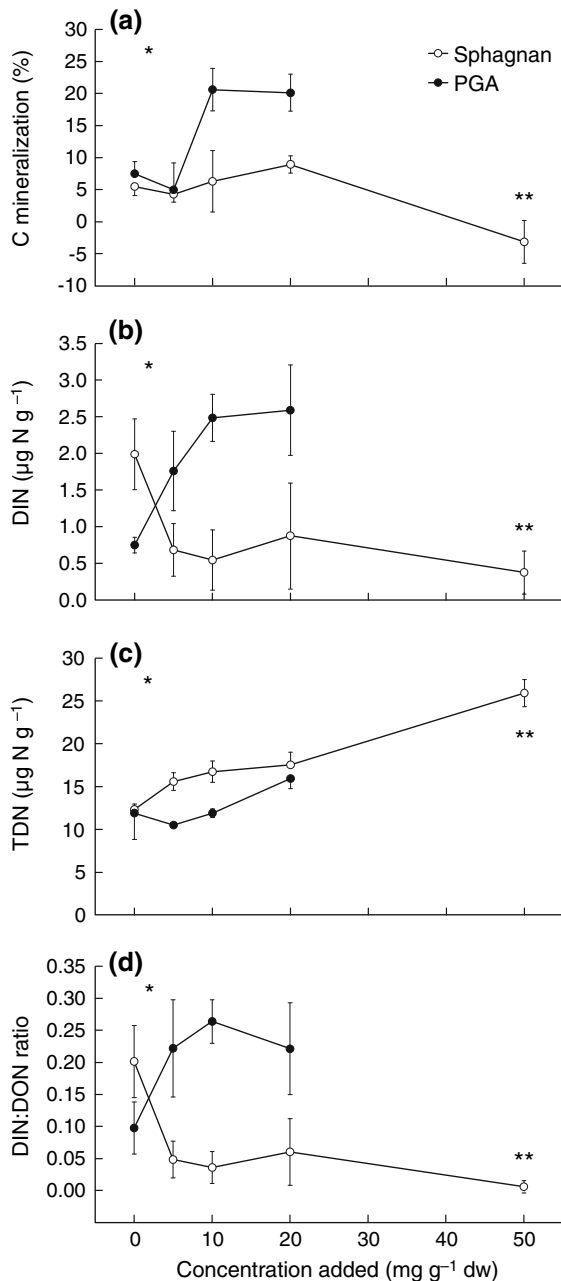| Source	Cumulative respiration			Respiration rates		
	F	d.f.	P	F	d.f.	P
Species	407.6	1	<0.0001	314.1	1	<0.0001
pH	73.9	2	<0.0001	69.4	2	<0.0001
Species × pH	14.3	2	0.0007	13.8	2	0.0008
TIME [repeated measures factor]				106.5	10	<0.0001
TIME × species				69.8	10	<0.0001
TIME × pH				4.0	20	<0.0001
TIME × species × pH				100.8	20	<0.0001

Fig. 4 Experiment 4. Effects of adding sphagnan and polygalacturonic acid (PGA) on **a** cumulative C mineralization of *Eriophorum angustifolium* leaf litter, **b** total dissolved N (TDN), **c** dissolved inorganic N (DIN), and **d** the ratio between DIN and dissolved organic N (DIN:DON). Error bars indicate ± SE. * = significant difference between sphagnan and PGA treatment (repeated measures ANOVA over first four concentrations, see Table 1), ** significant difference between adding 0 (control) and 50 mg sphagnan g^{-1} substrate (Student's t-test)

indirectly through the N-cycle and a decrease in DIN availability (experiment 4). In its cell-wall bound form, sphagnan is responsible for the high cation exchange capacity of *Sphagnum* that enables efficient nutrient acquisition under the extremely nutrient-poor and acidic environment of bogs (Clymo 1963; Hájek and Adamec 2009). Once released from the cell walls through autogenic acid-catalysed hydrolysis, sphagnan is stable (Painter 1991) and is not used as a C source (in contrast to PGA; experiment 4). Soluble sphagnan is a highly-branched macromolecule, its hairy appearance likely hampering easy access of hydrolysing enzymes. This recalcitrance of sphagnan is in contrast to non-branched de-esterified pectin chains, which generally undergo acid catalyzed hydrolysis easily (BeMiller 1986), as may be the case of the PGA used in our experiment 4 and of the lowered C-mineralization of *L. glaucum* after partial carboxyl reduction in experiment 2.

As stated in the introduction, sphagnan could affect the activity of the decomposer community by electrostatic immobilization of extracellular enzymes (Ballance et al. 2008) or lowering the pH (Stalheim et al. 2009). Ballance et al. (2008) studied interactions between isolated sphagnan and gelatine (a solubilised protein) at low ionic strength of 30 mM. Under low pH of about 2.0–4.8, sphagnan still had enough negative charge (pK = 3.5) to bind the positively charged gelatine. This resulted in coacervation, i.e., colloidization of a sphagnan-gelatine complex. Although our experiments were not designed to directly test this mechanism, results of the experiment 4 do not argue against it: sphagnan addition of 50 mg g^{-1} resulted in DON accumulation (as exoenzyme immobilization), DIN shortage (due to lower N mineralization resulting from the immobilization) and the decrease of C-mineralization (due to N limitation and/or exoenzyme immobilization). Increased pH in experiment 3 was followed by increased C mineralization in *Sphagnum* but not completely in *Leucobryum*. Although increased pH would likely disable coacervation by decreasing the positive charge of proteins, our experiment does not allow us to separate between the coacervation effect and other pH-related effects such as a shift towards the pH optimum of extracellular enzymes or towards a physiological optimum of the microbial community.

Table 4 Experiment 4: ANOVA results for factorial effects of substance addition (sphagnan or PGA) and its concentration (0, 5, 10 and 20 mg g^{-1}) on cumulative C mineralization, TDN, DIN, and DIN:DON

Source	d.f.	C mineralization		TDN		DIN		DIN:DON	
		F	P	F	P	F	P	F	P
Substance	1	5.20	0.06	7.40	0.03	5.90	0.04	7.30	0.03
Concentration	3	9.50	<0.01	6.20	<0.01	0.40	0.74	0.10	0.98
Substance × concentration	3	4.90	0.01	2.10	0.12	4.50	0.01	4.00	0.08

Perhaps the most intriguing result is that there are more biochemical alternatives in the plant world leading to slow litter decomposition. In some ecosystems, such as subtropical and forested peatlands, lignin-derived soil C dominates (Kuder et al. 1998; Williams et al. 1998; Jin et al. 2009), while in other ecosystems, such as *Sphagnum*-dominated bogs, polysaccharide-derived C prevails (Williams et al. 1998; Yavitt et al. 2005). But how can this result change our understanding of the environmental processes that control the rate of litter mineralization? Will the chemical composition and mineralization of polysaccharide-dominated litter respond differently from the lignin-rich litter to environmental changes, such as increased temperature, CO_2 concentration, aerial N and S deposition or water deficiency? Although the answers to these questions merit further research, it is likely that the biggest difference would be the sensitivity of the decomposer activity after water table draw down. In phenolics-dominated litter the shut-down of phenol oxidase under anaerobic water-logged conditions is an important latch on C-mineralization (Freeman et al. 2001) which is far less effective in polysaccharide-dominated litter (Kuder and Kruge 2001).

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