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Freeze-thaw cycles simultaneously decrease peatland photosynthetic carbon uptake and ecosystem respiration

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Decreasing snow cover in winter resulting from climate warming increases the incidence of freeze–thaw cycles (FTCs) in many ecosystems, including peatlands. As peatland ecosystems form a globally significant long-term carbon storage, understanding the effects of changing conditions in winter on carbon dynamics is essential. We studied how FTCs affect peatland carbon cycling by conducting mesocosm experiments with *Sphagnum*. Our results indicate an overall impeding effect of FTCs on *Sphagnum* photosynthesis, chlorophyll content, ecosystem respiration and enzymatic processes. A threefold reduction in photosynthesis in the FTC treatment was related to a decrease in chlorophyll content, showing that *Sphagnum* physiologically suffers from repeated FTCs. In the FTC treatment β-glucosidase and phosphatase enzymatic activities decreased by 50% and 30%, respectively, whilst alanine remained unaffected, indicating that in peat soils short-term FTCs affect the carbon and phosphorus cycles, but not the nitrogen cycle. Long-term effects of FTCs deserve further studies.

Introduction

The presence of snow cover in winter is important in many ecosystems, as its insulating properties regulate thermal conditions by disconnecting air and soil temperatures (Henry 2008, Wright *et al.* 2009, Robroek *et al.* 2013). Decreasing amounts of snowfall and increased winter warm-

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ing have been predicted to result in shallower, sometimes even periodically absent snow cover, especially in the temperate zone of the northern hemisphere (Jylhä *et al*. 2004, Moss *et al*. 2010). This will result in increased frequency of soil freeze-thaw cycles (FTCs) (Bombonato and Gerdol 2012), a phenomenon often referred to as 'colder soils in a warmer world' (Groffman *et al*. 2001). Freeze–thaw cycles have been shown to affect nutrient availability and the community structure of vegetation and below-ground microbes in many ecosystems (Henry 2008, Kreyling *et al*. 2010, Kreyling and Henry 2011, Templer 2011), including peatlands (Bokhorst *et al*. 2008, Robroek *et al*. 2013, Jassey *et al*. 2016). Although the effect of climate warming and concurrent FTCs on peatlands were studied before (e.g. Wright *et al*. 2009, Wang *et al*. 2014), the simultaneous effect of FTCs on above-ground (e.g. *Sphagnum* photosynthesis) and below-ground (e.g. microbial respiration) processes, to our best knowledge, remains overlooked. In particular, as above- and below-ground processes are interlinked in peatlands and both contribute to the carbon cycle and to net carbon uptake, the simultaneous effect of FTCs on both compartments of the ecosystem needs further attention.

Peatlands store vast quantities of carbon as partially degraded organic matter (i.e. peat) due to the imbalance between decomposition and productivity. The carbon sink function of peatlands is particularly sensitive to changes in climatological conditions (Coulson and Butterfield 1978, Turunen *et al*. 2002, Laiho 2006). Therefore, more frequent FTCs as an indirect effect of climate change may have an important role in the potential shift of peatland ecosystems from carbon sink to carbon source (Bombonato and Gerdol 2012). *Sphagnum* mosses are crucial in the process of carbon accumulation in peatlands. Their productivity is known to be dependent on a range of biotic and abiotic conditions (e.g. Gunnarsson 2005), yet the role of FTCs is generally overlooked. Parallel to the impeding effect of freeze–thaw cycles on vascular plants (e.g. Min *et al*. 2014), FTCs are likely to damage *Sphagnum* due to freezing and subsequent quick thaw, as the tolerance of lipid bilayers forming cell membranes to compression and stretching is very limited (Schmitt *et al*. 1985). This could strongly reduce carbon assimilation in the system and thus impact peatland carbon balance. Moreover, *Sphagnum* mosses are associated with diverse microbial communities that govern decomposition processes (Bragina *et al*. 2014). Hence, if *Sphagnum* is damaged by FTCs, microbes living in *Sphagnum* mosses are also likely to be affected by FTCs and their functions altered (Jassey *et al*. 2016). Robroek *et al*. (2013) showed that snow removal affects microbial community structure, potentially causing changes in microbial processes like enzymatic activity. Indeed, most enzymes are proteins and can therefore be affected by changes in chemical environment (e.g. pH) and temperature (Puissant *et al*. 2015). Although changes in enzymatic activity can cascade to important functions like the carbon cycle, data on the effect of winter climate change on peatland microbes is very scarce (but *see* Tsyganov *et al*. 2012 and Jassey *et al*. 2016).

Here we postulate that freeze–thaw cycles force *Sphagnum* and microbial communities to repeatedly acclimatise to the rapid change in temperature. As such, freeze–thaw cycles are an environmental stress that could impact the photosynthetic capacity of the peat mosses and the functioning of the microbial community. FTCs have been shown to decrease chlorophyll concentrations in plants (Deltoro *et al*. 1999, Zhang *et al*. 2014); we therefore hypothesize (i) that *Sphagnum* chlorophyll content decreases with repeated FTCs, further decreasing *Sphagnum* photosynthetic capacity and carbon uptake of the system. Microorganisms are usually highly sensitive to deep soil freezing and many taxa have no tolerance to freezing (Schimel and Mikan 2005, Walker *et al*. 2006, Yergeau and Kowalchuk 2008); we thus hypothesize (ii) that FTCs reduce respiration and enzymatic activity in ombrotrophic peatlands.

Materials and methods

Bryophyte material

In mid-February 2015, we collected 16 mesocosms (diameter 7.5 cm, depth 15 cm) from

the ridge-hollow ecotope (Küttim *et al*. 2016) in Männikjärve raised bog (Estonia), where the mean long-term (1962–2012) annual temperature and precipitation are 5.1 °C and 710 mm, respectively (Estonian Weather Service). The average daily maximum of solar radiation at the Männikjärve bog (58°52´N, 26°14´E), during midwinter is 55 W m^{-2} (but on some days only 8 W m^{-2}), and about 500 W m^{-2} in early spring, when the snowpack is decreasing and thereby FTCs occur (Estonian Weather Service). Mesocosms were taken in pairs from eight patches whose diameter was ≥ 40 cm, and coverage of *Sphagnum magellanicum* ≥ 95%. The bottom of each mesocosm was covered with nylon mesh (100 μm) so as to prevent any loss of peat material, but allowing excess water to drain. After collection, the mesocosms were kept at 5 °C until the start of the experiments.

Experimental set-up

One mesocosm from each pair was assigned to a control (CON), and the other to experimental freeze-thaw cycles (FTC), resulting in eight control and eight FTC mesocosms. All mesocosms were placed in PVC boxes (32 × 37 × 56 cm). To mimic natural conditions where only the surface is exposed to frost, the sides and the bottom of each mesocosm were insulated with styrofoam (the bottom plate was perforated to allow water to drain). Air temperature (10 cm above the capitula) and peat temperature (2 cm below the capitula) were measured at 30-minute intervals using Decagon ECT temperature probes connected to an Em50 data logger (Decagon Devices Inc., Pullman, WA, USA).

Our experimental design consisted of two parallel experiments. In the first experiment, the FTC mesocosms (n = 4) were subjected to seven cycles of above- and sub-zero air temperatures for one week in a dark incubator. Specifically, during daytime (thawing phase: 08:00–20:00 hrs) the air temperature was set to +5 °C, whilst during the night (frost phase: 20:00–08:00 hrs) it was set to –5 °C. In the second experiment, the FTC mesocosms (n = 4) were kept outside at the ambient temperature and light (~500 W m^{-2}) during daytime (thawing phase: 08:00–18:00 hrs) and then transferred to an incubator where the temperature was kept at –5 °C (frost phase: 18:00–08:00 hrs. Both experiments were carried out at the École Polytechnique Fédérale de Lausanne, Switzerland (46°31´N, 06°38´E).

Control mesocosms (n = 4, for each experiment) were kept in a dark incubator at +5 °C, as this has been reported to be the threshold for active *Sphagnum* photosynthesis (Gerdol *et al*. 1996, Haraguchi and Yamada 2011). All mesocosms were watered with 100 ml of distilled water before, during and after the treatments to keep mesocosm water content above 90% (Thimonier *et al*. 2005).

The FTC mesocosms in the first experiment did not experience any freeze–thaw cycles: instead they remained frozen throughout the experimental period (Fig. 1a). This was probably due to the absence of solar energy. As a result, they were excluded from the analysis and only the mesocosms form the second experiment where clear FTCs were recorded (Fig. 1b) were considered.

Measurements and analyses

Sphagnum photosynthesis

Photosynthetic capacity (A_{max}) of *S. magellanicum*, defined as the maximum CO_2 assimilation rate under optimum light conditions, was measured at the capitulum level (reducing the possible measure of CO_2 loss from non-photosynthetic tissue) at the end of the experimental treatment. Capitula (n = 2–3) were harvested together, 30 minutes after rewetting. Measurements were performed using an open infrared gas analyser (IRGA) system connected to a 2.5 cm^2 PLC-6 chamber (CIRAS-2, PP-Systems, Amesbury, USA). Initial pilot measurements (data not presented) indicated an optimum light level for *S. magellanicum* photosynthesis at a PPFD of 600 μmol photons m^{-2} s^{-1} at 20 °C. The environmental conditions inside the cuvette were controlled and kept constant during the measurements with the leaf temperature and CO_2 concentration being 20 ± 1 °C and 380 ± 2 ppm, respectively; the relative humidity was always

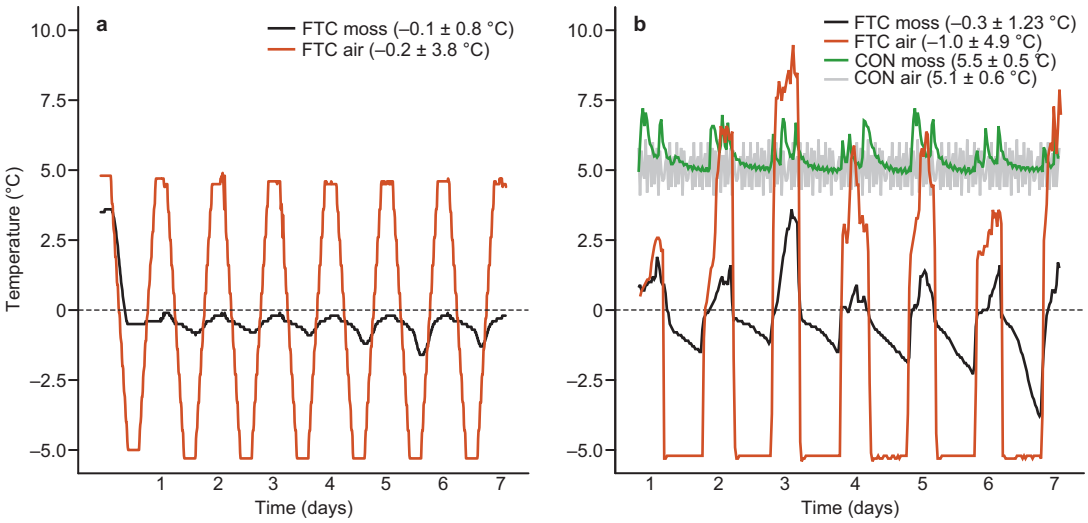

Fig. 1. FTC and CON air temperatures and moss temperatures at 2 cm depth below the capitula in (**a**) the first and (**b**) second experiments. Mean temperatures ± SE for each treatment are given in the legends.

close to ambient conditions. Capitula were weighed directly after gas measurements and subsequently dried at 65 °C for 48 h to constant weight (DW). A_{max} is expressed as mg of CO_2 per gram of DW per hour (mg g^{-1} h^{-1}). All moss water contents were above 90%.

Chlorophyll concentration

Chlorophyll-*a* and -*b* concentrations were determined after the experimental treatments, by extracting the green tissue pigments from *S. magellanicum* capitula in 96% ethanol (Lichtenthaler 1987). Freeze-dried samples were cut into small pieces using scissors and homogenised. Next, 5 mg of dry tissue was hydrated with 100 μl of distilled water. After 10 minutes, chlorophyll was extracted in 8.0 ml of 96% ethanol at room temperature over night. The following day, samples were vortexed and centrifuged, after which absorbance of the supernatant was measured on a spectrophotometer at 470 nm, 648.6 nm, 664.2 nm and 750 nm (Shimadzu, UV-120-01, Japan).

Respiration

Measurements of the CO_2 flux in the FTC and CON treatments were made using a LI-COR LI-8100 automated soil CO_2 flux system (LI-COR Inc., Lincoln, Nebraska, USA). Throughout the seven-day experimental period, we performed dark-chamber (1140 cm^3) measurements three times per day (i.e. 9:00, 12:00 and 17:00). Note that all the measurements were made during the day (the thawing period in the FTC treatment) and, hence, under similar conditions for both FTC and CON cores. Further, respiration included respiration from mosses, moss endophytic microbes and soil microbes. Chamber measurements were started after the first frost period, and were comprised of a 2-minute closure time during which headspace CO_2 concentrations were recorded 120 times. Fluxes were then calculated from the changes in the chamber headspace CO_2 concentrations in time, using linear regression.

Potential enzyme activity measurements

Potential enzyme activity assays were conducted as described in Jassey *et al.* (2011, 2016). We used substrates labelled with the fluorophores 7-amino-4-methylcoumarin (MUC) or methylumbelliferone (MUB) to quantify the relative activity (i.e. enzyme activity under saturating substrate conditions) of enzymes responsible for the hydroly-

sis of one peptide [L-alanine-7-amido-MUC, Alanine-aminopeptidase (ALA)], one carbohydrate [4-MUB-β-glucopyranoside, β-glucosidase (BGA)] and one phosphate ester [4-MUB-phosphate, acid phosphatase (AP); all substrates supplied by Sigma-Aldrich Switzerland]. Enzymes were analysed in microplates using slurries created by homogenizing 3 g fresh weight of soil. Briefly, 3 g fresh weight *S. magellanicum* shoots (0–5 cm depth) per mesocosm were ground with 50 ml of a 0.1 M $CaCl_2$ solution with 0.05% Tween 80 and 20 g of polyvinylpoly-pyrrolidone, and shaken at room temperature for 1.5 h on a reciprocal shaker (150 rpm). The resulting suspension was filtered to remove the largest floating particles, and then centrifuged at 5000 rpm for 5 min at 4 °C. After successive filtration of the supernatant through 1.2 μm Whatman GF/C filters, the extracts were concentrated in cellulose dialysis tubes (Medicell International Ltd., London, UK) with a 10 kDa molecular mass cut-off, covered with polyethylene glycol (PEG) until approximately 1/10 of the initial volume. The extract was re-suspended to 10 ml with phosphate buffer (pH 5.6) and separated in two equal fractions. To correct enzymatic activities, one fraction was stored at 4 °C, the other was boiled for 3 h at 90 °C and used as a control.

For each sample, we used four methodological-replicate assay wells receiving 38 μl of enzymatic extract and 250 μl of substrate. Four other methodological-replicate assay wells were filled with 38 μl of boiled enzymatic extract and 250 μl substrate as control. Incubations were performed at 25 °C for 3 h after which the reactions were stopped with 1 μl NaOH (0.5 M). Fluorescence was monitored spectrophotometrically with an excitation wavelength of 365 nm and emission detection at 450 nm (Biotek, SynergyMX). All measurements were converted to nanomoles per gram dry weight per min (nmol g^{-1} min^{-1}).

Data analysis

Prior to statistical analyses, data were tested for normality by Kolmogorov-Smirnov test, and for equality of variance by Levene's test. All data were normally distributed and had equal variances. Respiration rates were averaged per mesocosms which were then used for testing the effect of FTCs on respiration rates. The effects of FTCs on A_{max}, chlorophyll concentration, respiration rate and enzymatic activity were tested using paired *t*-test. Linear regressions were computed to analyse the relationships between A_{max} and chlorophyll-*a* concentration, respiration and temperature, and respiration and enzyme activities. Throughout the paper, treatment means with their standard errors are presented. All analyses were performed by IBM SPSS 20 Statistics software.

Results

Photosynthetic capacity (A_{max}) of mosses in the FTC experiment was approximately three times lower than that of the control ($t_3 = -1.7$, $p \leq 0.001$), and there was a decline in *Sphagnum* chlorophyll-*a* concentration ($t_3 = -2.7$, $p \leq 0.001$; Fig. 2). A_{max} and chlorophyll-*a* concentration correlated across treatments and in both treatment groups (FTC: $R^2 = 0.85$, $n = 4$, $p = 0.043$; CON: $R^2 = 0.79$, $n = 4$, $p = 0.014$).

Respiration rates in the mesocosms in FTC experiment were considerably lower ($t_3 = 9.6$, $p \leq 0.001$) than those in the control (on average, FTC = 0.09 ± 0.07; CON = 0.60 ± 0.21 μmol m^{-2} s^{-1}; Fig. 3). Although respiration rate tend to decrease throughout the experiment in both treatments, no daily pattern occurred, and the respiration in FTCs was always lower than in controls. Additionally, the rate of respiration was significantly affected by temperature within the mesocosms ($R^2 = 0.38$, $n = 4$, $p \leq 0.001$).

The responses of potential enzyme activities to FTCs were enzyme-specific (Fig. 4). BGA (–50%; $t_3 = -2.7$, $p = 0.033$) and AP (–30%; $t_3 = -8.9$, $p \leq 0.001$) were markedly lower in FTCs than in the controls, while ALA remained unaffected ($t_3 = 0.05$, $p = 0.264$). Decreases in enzymatic activities most likely underlay the decrease in respiration rates under freeze–thaw conditions as mean respiration rates correlated with BGA ($R^2 = 0.56$, $n = 4$, $p \leq 0.001$) and AP activities ($R^2 = 0.81$, $n = 4$, $p \leq 0.001$). There was no correlation between ALA and respiration ($p = 0.271$).

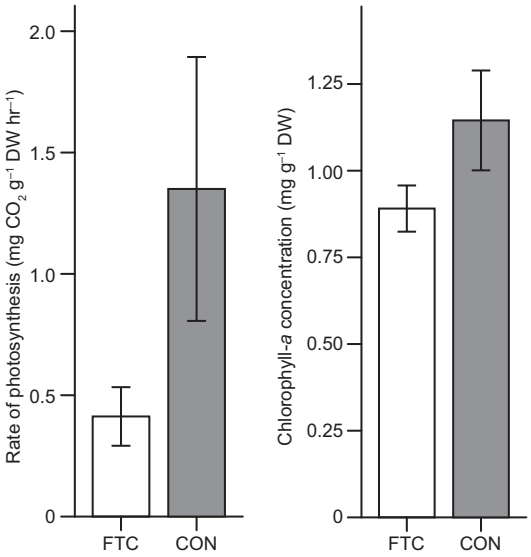

Fig. 2. Photosynthetic capacity (A_{max}) and chlorophyll-*a* concentration of control (CON, kept at +5 °C) and FTC-treated *Sphagnum magellanicum* (FTC, kept at ambient outdoor temperature and –5 °C during day and night, respectively) at the end of the experimental treatments. Error bars represent standard errors.

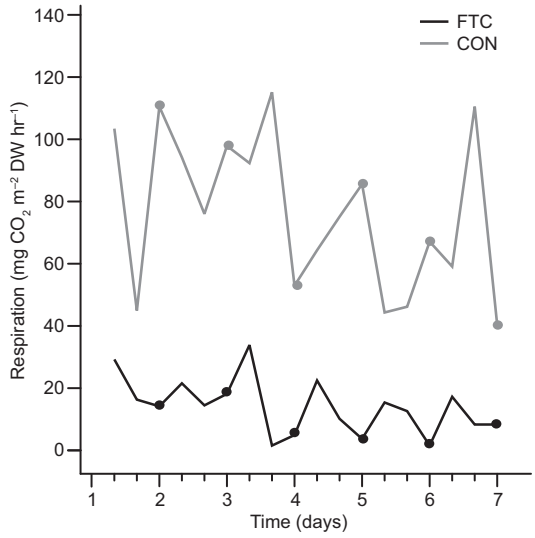

Fig. 3. Mean respiration rates for CON and FTC moss mesocosms (FTC) measured at 09:00, 12:00 and 17:00, and throughout the experiment. The first measurements of each day at 09:00 is indicated by a black dot.

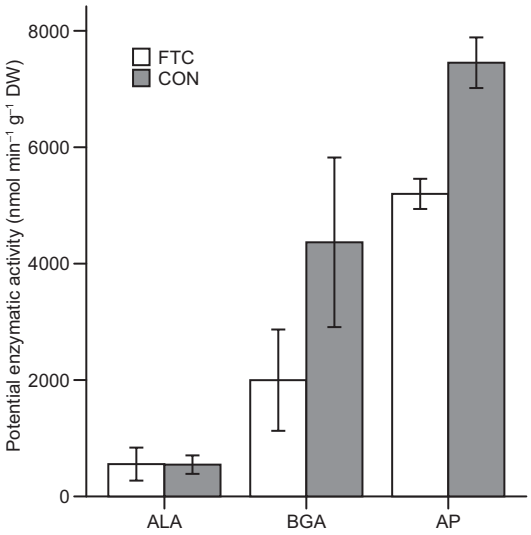

Fig. 4. Potential enzymatic activity of alanine-aminopeptidase (ALA), β-glucosidase (BGA) and acid phosphatase (AP) of control (CON, +5 °C) and FTC-treated mosses (FTC, ambient and –5 °C) at the end of the experimental treatments. Error bars represent standard errors.

Discussion

Projected climate change is expected to increase the frequency of soil freeze-thaw cycles (FTCs) (Bombonato and Gerdol 2012). Our experiments revealed that merely positive daytime temperature is not enough to thaw the frozen peat, but that energy form solar radiation is instrumental. These seem obvious, but in many laboratory studies of the effects of FTCs on plant performance dark incubators are used (e.g. Min *et al*. 2014). Our results highlight the necessity of additional energy (either solar, or artificial IR lamps) to induce cyclic (24 h) freeze–thaw events, at least in peat soil.

As hypothesized, photosynthetic capacity in *S. magellanicum* was substantially lower when it was subjected to freeze–thaw cycles. These results underpin that peat moss carbon uptake through photosynthesis is impeded by repeated freezing. While mosses tolerate freezing much better than do most vascular plants (Glime 2007), FTCs are considered one of the most severe environmental stressors on moss performance (Kennedy 1993, Deltoro *et al*. 1999). The decrease in photosynthetic capacity caused by FTCs may be the result of (1) a direct effect on the moss physiology, e.g. through changes in pigment concentrations, (2) mechanical stress due to formation of ice crystals (Schmitt *et al*.

1985, Kennedy 1993), or (3) an increase in the concentrations of cellular solutes due to cell dehydration (Schmitt *et al*. 1985). Our results show that the lower photosynthetic capacity under freeze–thaw conditions may be due to a decrease in chlorophyll-*a* concentration. Gerdol *et al*. (1994) showed that chilling can trigger a rapid degradation of chlorophyll. In addition, interaction between solar radiation and frost can amplify the effect on chlorophyll *a* (Glime 2007) resulting in a smaller absorbance of light energy (Huner *et al*. 1993).

Snow, because of its insulating properties, keeps below-ground temperatures rather constant, and allows for microbial metabolism during winter (Brooks *et al*. 1997, Bombonato and Gerdol 2012). Therefore, winter CO_2 fluxes in peatlands should not be ignored, as they can form 8%–14% of annual CO_2 release (e.g. Leppälä *et al*. 2011). Soil respiration rates in our control mesocosms were comparable to those measured in the earlier studies (Alm *et al*. 1999, Kim *et al*. 2007, Leppälä *et al*. 2011). Reduced snow cover and concurrent FTCs could however result in microbial biomass reductions, ultimately lowering ecosystem respiration rates (Larsen *et al*. 2002, 2007). Supporting our hypothesis, a threefold reduction of mean respiration rate was observed after the mesocosms experienced FTCs. Larger decline in respiration in the FTC than in CON mesosoms in the course of the experiment suggest that this reduction was not caused by a mere change in temperature, but that respiration rates were hampered by freeze-thaw cycles.

Although Robroek *et al*. (2013) obtained similar results from a Swiss mountain bog, we currently lack consensus on the effect of FTCs on microbial respiration. While we found a decrease, some studies report only minor effects of FTCs on microbial respiration in sub-arctic (Larsen *et al*. 2007), alpine (Bombonato and Gerdol 2012) and boreal (Johansson 2010) peatlands. Possibly, prevailing environmental conditions and adaptation of the microbial communities to those conditions play an important role in their response to soil frost. Nevertheless, the effects of freeze–thaw cycles on soil microorganisms are still not well understood (Kreyling and Henry 2011). We found an overall temporal reduction in respiration rates in both FTC and CON treatments, which could be due to experimental conditions. There is evidence that freeze–thaw cycles strongly reduce the activity and population dynamics of microorganisms in soils because repeated fluctuations in temperature can damage or even destroy microbial cells (Schimel and Mikan 2005, Walker *et al*. 2006), and hence decrease microbial activity. Also Schimel and Clein (1996) recorded a decreasing respiration rate after every FTC, and related it to a parallel decline in living microbial biomass, as a significant amount of microbes were killed by each cycle.

We found that lower respiration rates in response to FTCs were related to lower enzyme activity, except ALA that remained unaffected by FTCs. The latter finding supports those by Weedon *et al*. (2012, 2014), who demonstrated that all potential peptidase activities were unaffected by temperature. ALA, and probably other peptidases as well, appear to be less sensitive to FTCs than AP and BGA, and in addition, peptidase activity in peatlands is rather minimal (Weedon *et al*. 2014). This points to a minor effect of FTCs on N cycle in peatlands, as peptidases are responsible for the cycling of organic N forms. Peptidases are more active in the beginning of the summer (Weedon *et al*. 2014) and therefore have to be somewhat less dependent on FTCs that rarely occur after May in boreal peatlands. AP participates in the mineralization of phosphate (Dodd *et al*. 1987, Rodriguez and Fraga 1999) and decreased about 30% in FTC relative to CON. If FTCs are long-lasting and severe, a significant part of the soil microbial component could be exacerbated and therefore produce less extracellular enzymes, which in case of AP leads to a reduced amount of available phosphate. Such scenario may imbalance the stoichiometry of available nitrogen and phosphorus.

Among the tested enzymes, BGA showed the strongest response to FTCs. Further, potential enzymatic activity of BGA decreases along with ecosystem respiration rate, which support previous findings on BGA activity as a proxy for carbon respiration (Sinsabaugh *et al*. 2008). Taken together, our results highlight that FTCs reduce carbon release from peatlands at least

in the short term. When microbes are exposed to cold temperatures, the microbial community most likely shifts into a cold-adapted state, where microbes synthesize enzymes that work in low temperatures. It is therefore possible that a functional isozyme replacement occurs, resulting in an adaption of decomposition process to low temperatures (Blagodatskaya *et al*. 2016).

Reductions in AP and BGA potential activities after soil FTCs are related to reduction in microbial biomass or activity. Although microbes can survive and grow with temperatures below zero (Gilchinsky and Wagener 1995, Alm *et al*. 1999), repeated crossing the 0 °C threshold leads to direct shifts in microbial activity. This is due to lower availability of liquid water (Mikan *et al*. 2002, Kreyling and Henry 2011) that hinders the diffusion of extracellular enzymes (Elberling and Brandt 2003). Microorganisms that accumulate osmolites to survive the cold temperatures could possibly die during thawing of the soil because of an osmotic shock caused by the sudden availability of melt water from snow (Jefferies *et al*. 2010). This can be coupled to changes in microbial community structure and affect the overall functioning of the ecosystem (Larsen *et al*. 2002). Although we did not test the recovery speed of photosynthetic and microbial activity after FTCs, it has to be noted that microbial organisms react more rapidly to changing environmental conditions than macroorganisms (Hajek *et al*. 2011). However, the effects of FTCs on annual ecosystem carbon accumulation remain subject to further study.

Conclusions

Our experiment confirmed that diurnal FTCs impede photosynthesis and microbial enzymatic activity and concurrent ecosystem respiration. The increase in frequency and severity of FTCs may also affect vegetation and microbial structure, nutrient content and stoichiometry, and the phenology of many species. Thus the functioning of peatland ecosystems in the winter may be slowed down due to decreasing snow cover and concurrent FTCs. Although in the short term the changes in carbon uptake processes are roughly balanced by carbon release reductions, the long-term effects remain unknown. However, in the severe and long-lasting FTC cases these effects might cascade to the growing season and lower biomass production.

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