Chapter 5

Substrate-driven indirect effects dominate the apparent temperature sensitivity of peatland C and N cycling

5.1 Abstract

Northern peatlands constitute an important component of the global carbon (C) cycle due to their long-term accumulation of carbon as soil organic matter. This function as a carbon sink is partly dependent on low temperatures limiting decomposition and nutrient cycling, so global warming has the potential to alter the C balance of these systems and feedback to climate change. Field observations have shown that organic matter decomposition, ecosystem respiration and nitrogen cycling are intertwined processes that show disproportionate temperature sensitivity. In the current study, we investigate whether seasonal dynamics of substrate input may represent an indirect mechanism for these observed patterns. To this end, we carried out a 60-day mesocosm incubation experiment with sub-arctic peat soil to compare the direct effects of temperature increase with the indirect effects of increased microbial- or plant-derived organic matter input on key soil C and N cycling processes and substrate pools. Additions of dead microbial cells led to increases in N pool sizes and the potential activities of most enzymes, and a transient increase in the relative abundance of β-proteobacteria, and decrease in the relative abundance of α-proteo-, Actino- and Acidobacteria. Neither the addition of plant root litter, or a 5 °C alteration in incubation temperatures had comparable effects on these parameters. Peat respiration was positively affected by both substrate addition (20-46% increase) and higher incubation temperatures (34-38% increase), but the temperature-only effect was not sufficient to account for the increases in respiration observed in field experiments. Thus, it appears that warming effects on C and N cycle processes are dominated by indirect effects, acting through alterations to the seasonal flux of microbe-derived organic matter. High apparent temperature sensitivity of decomposition and respiration may therefore be caused by changes to substrate supply rates. We propose that climate change models of soil carbon and nitrogen cycling should explicitly incorporate realistic microbial biomass dynamics.
5.2 Introduction

Predicting future climate scenarios requires a thorough understanding of the global carbon (C) and nitrogen (N) cycles, and the nature of the feedbacks between biogeochemical processes and climate variables (Heimann & Reichstein, 2008). Northern peatlands are a particularly important component of these cycles, given their role as a large C sink over the last 12,000 years (Adams et al., 1990; Limpens et al., 2008). In these environments, organic matter decomposition is constrained by combinations of low pH, low temperatures, functionally limited decomposer communities, oxygen limitation of oxidase activity, and chemically complex substrates with generally low nitrogen content (Freeman et al., 2001; Moore & Basiliko, 2006). These constraints have led to a long-term accumulation of organic matter - peatlands currently contain 15 to 30% of the global soil C pool, despite covering only 3% of the Earth’s terrestrial surface area (Gorham, 1991; Turunen et al., 2002). This large C store is potentially vulnerable to changes to climate which alleviate any of the current constraints on decomposition (Gorham, 1991). Climate change due to greenhouse gas emissions is predicted to raise mean global temperatures by 1.0-3.5 °C in the next 50-100 years with above-average increases at high-latitude and high-altitude regions (ACIA, 2004; IPCC, 2007). This increase in temperature may directly affect both productivity and decomposition in these areas (via thermokinetic effects), but also indirectly through effects on soil N availability (Rustad et al. 2001). Such an indirect effect may be particularly important given that both production and decomposition are usually N-limited in these systems. Thus, changes to N availability may have strong effects on the ecosystem C balance (Aerts et al., 2001; Bragazza et al., 2006).

Several experimental and observational studies provide evidence that peatland ecosystem processes are particularly sensitive to changes in temperature. Eddy covariance measurements in a Canadian forested peatland indicated that photosynthesis and respiration are both stimulated by warmer conditions (Flanagan & Syed, 2011). A mesocosm experiment using peat monoliths from contrasting fen and bog environments found that ecosystem respiration was mostly driven by temperature, and that, over the long-term, increased temperatures altered soil quality with consequences for CO₂ and CH₄ emission patterns (Updegraff et al., 2001; Keller et al., 2004). At a larger scale, studies in peat bogs across a continental gradient suggest that changes to C and N dynamics with increasing temperature will mainly be driven by increases in vascular plant production (Breeuwer et al., 2008; Breeuwer et al., 2010). In a long-term seasonal climate manipulation experiment at an acidic, ombrotrophic bog in Abisko, Sweden, spring and summer warming of only 1-1.5 °C increased the production of the dominant Sphagnum moss species (Dorrepaal et al., 2004; Keuper et
al., 2011) and stimulated ecosystem respiration (Dorrepaal et al., 2009) by around 60 and 52%, respectively. At this site, we have also observed unexpectedly large increases in the fluxes of organic nitrogen and ammonia in summer-warmed plots (c. 100% greater than controls). Given the modest experimental temperature increase, it is very unlikely that this was a direct temperature effect (which would imply a $Q_{10}$ value for N mineralization of c. 30). Therefore, indirect effects, such as the observed non-specific dieback of the microbial biomass over the summer growing season (Chapter 3) seem to play an important role.

This surprising result is linked to the intensive study and debate in recent years about the factors controlling the temperature sensitivity of different ecosystem processes, particularly soil organic matter (SOM) decomposition (Conant et al., 2011). One aspect of the discussion has centered on whether decomposition of recalcitrant SOM is more or less temperature sensitive than that of labile SOM (Giardina & Ryan, 2000; Conant et al., 2008; Craine et al., 2010). In addition to these “SOM quality” effects — which are to some extent predictable from Arrhenius kinetics (Davidson & Janssens, 2006) — it has been recognized that apparent temperature sensitivities can be greater or lesser than the intrinsic temperature sensitivity of the process due to interactions between temperature and other constraints on process rates, such as physico-chemical conditions and/or substrate supply (Davidson & Janssens, 2006). For example, if warming relaxes an environmental constraint on decomposition, then observed apparent temperature sensitivities may be much larger than the intrinsic temperature sensitivity of the substrate (Figure 5-1). Such effects have been observed and/or predicted when warming leads to shifts from anoxic to oxic soil conditions (Freeman et al., 2001), changes to soil substrate diffusion processes (Davidson et al., 2011), or alterations in the equilibria of adsorption/desorption interactions of dissolved organic matter (Conant et al., 2011). Furthermore, temperature increases may lead to the crossing of important (a)biotic thresholds such as soil thawing (Deluca et al., 1992; Jefferies et al., 2010) and initiation of microbial activity (Chapman & Thurlow, 1998). Although all of the above processes can play a role in generating high apparent temperature sensitivities, we propose that temperature-driven increases of labile substrate inputs into the soil system may also be important. If such substrate inputs lessen the degree of substrate limitation of enzymatic reactions then temperature-related flux increases above the theoretical intrinsic temperature sensitivity of a process may be observed.

It is commonly observed that warming increases the input of substrates into the soil, via above- and below-ground litter production, and/or turnover of microbial biomass. A multi-site warming experiment across 11 high-latitude sites showed a consistent positive effect of warming on plant litter production (Walker et al., 2006), as did a
Figure 5-1: Conceptual model of how indirect temperature effects can increase apparent temperature sensitivities above theoretical expectations. A hypothetical biological process (e.g. decomposition) has a reaction rate monotonically related to ambient temperature due to thermokinetic effects (dashed grey line), yet experiences a temperature-related indirect constraint on the process rate which leads to threshold dynamics (solid grey line). Paired observations across a (natural or imposed) temperature gradient may result in an apparent temperature sensitivity (solid black line) which is greater than the sensitivity expected based on thermodynamic principles (dashed black line).

Five-year temperature, water and nutrient manipulation experiment on a sub-arctic tundra in Northern Sweden (Press et al., 1998). Such increased inputs of plant-derived organic matter may lead to higher rates of ecosystem respiration and nutrient cycling. An additional source of extra substrate into the soil could be the turnover of the soil microbial biomass. In soils subject to seasonal thaw, the shift from winter to spring conditions causes large transient pulses of relatively labile nitrogen rich organic matter as the cold-adapted over-winter microbial community experiences a population crash and is replaced by a faster-cycling summer community (Deluca et al., 1992; Larsen et al., 2007; Schmidt et al., 2007). The degree to which this freshly released organic matter is re-immobilized in microbial biomass has important implications for the nutrient economy of the plant-soil system (Zak et al., 1990; Brooks et
al., 1998). Warming-related amplifications of this normal seasonal turnover of microbial biomass may lead to higher measured rates of soil organic matter transformations (Chapter 3, Schmidt et al., 2007) and may explain the high apparent temperature sensitivity of soil processes (von Lützow & Kögel-Knabner, 2009).

An important challenge in interpreting higher than expected apparent temperature sensitivities of C and N cycling from field observations is the difficulty in differentiating between direct and indirect mechanisms. We therefore conducted a mesocosm incubation experiment to separate the direct effects of temperature and indirect effects of increased substrate supply on a range of peat processes. We incubated peat mesocosms at three temperatures to quantify the direct temperature effect on respiration, nitrogen pool sizes, soil enzyme pool sizes, and microbial community structure—these latter two components being the proximate drivers of organic matter transformations in soil (Schimel & Gulledge, 1998; Allison et al., 2007). To compare the direct temperature effects with the effects of increased substrate supply, and the possible interactions between the two factors, we also imposed treatments that simulated an increase in inputs of either belowground plant litter or microbial biomass. This allowed us to answer the following questions:

1. What is the magnitude and direction of the direct temperature effect on peat respiration, nitrogen pool sizes, microbial community structure and potential enzyme activities?

2. How does a simulated pulse of plant litter-derived or microbe-derived organic matter affect the above parameters, and how do the magnitudes of these effects compare to pure temperature effects?

3. Assuming that warming under field conditions leads to both direct temperature effects and indirect effects via increased substrate supply, and based on the answers to 1) and 2), what inferences can we make about the relative importance of direct versus indirect effects of temperature on peatland C and N cycling?

5.3 Materials and Methods

5.3.1 Soil collection and preparation

Material for the mesocosm experiment was collected from the Stordalen mire complex (68°21'N 19°02'E) near Abisko, sub-arctic Sweden in late-April 2010. A single block of peat was cut out of a Sphagnum fuscum dominated area with scattered vascu-
lar plants (mostly Empetrum hermaphroditum, Andromeda polifolia, Rubus chamaemorus, Betula nana and Vaccinium vitis-idaea). The peat was cut to approximately 15 cm depth and was immediately frozen at -20 °C and transported to Amsterdam where it remained frozen until the beginning of the experiment three months later. One day before the beginning of the experimental incubation, the block was removed from the freezer and allowed to thaw at room temperature. The peat soil was then thoroughly cut into fragments < 2 cm length and all large vascular plant roots and leaf litter were removed.

5.3.2 Experimental set-up and incubation

The experiment consisted of a factorial combination of three temperature and three substrate treatments, each replicated four times, for a total of 36 experimental units. Approximately 200 g of homogenized peat (fresh weight, equivalent to 40 g dry weight) was added to each of 36 500 ml Schott bottles. Four bottles were randomly assigned to one of each of the 9 combinations of substrate and temperature treatments. The temperature treatments consisted of incubation at a constant temperature of 9, 11 or 14 °C, henceforth Low, Medium and High temperature treatment, respectively. 9 °C was chosen as it is the daily average temperature at 5 cm soil depth from June to August at the subarctic field site from which the peat had been collected (unpublished data). The other temperatures were chosen to simulate the experimental warming treatment (+2 °C) imposed under OTC experiments (Dorrepaal et al., 2004) and a larger temperature increase (+ 5 °C) that represented the predicted temperature increase for the end of this century at northern high latitudes (IPCC 2007). The three substrate treatments were Control – no substrate added, Cells – freshly killed $^{13}$C labeled bacterial cells added as substrate, and Roots – homogenized $^{13}$C labeled vascular plant roots.

The Cells substrate addition was prepared by culturing Pseudomonas sp. (strain PS+; DSM 12877) in 500 ml Pseudomonas basal mineral medium (Atlas & Snyder, 2006) with 50 ml 0.8M glucose solution (0.04% $^{13}$C) as carbon source. Cultures were incubated at 30 °C and once a stationary phase was attained, cells were concentrated by centrifugation, the pellet washed twice with dilute potassium phosphate buffer and then resuspended in sterile demineralized water at 5% of the original culture volume. Cells were then killed by heating to 80 °C for one hour. Total carbon and nitrogen content of the dead cell mixture was measured on an NA 1500 Elemental Analyzer (Carlo Erba, Italy), and the dead cell mixture was diluted with sterile demineralized water such that each incubation bottle in the treatment received 1.8 mg C g$^{-1}$ DW.
peat in 10 ml solution. This amount is equivalent to 50% of the peak microbial biomass N under summer warming in a nearby peatland (Chapter 3). The Roots substrate addition was prepared by grinding dried Carex rostrata roots from plants grown in $^{13}$C enriched CO$_2$ atmosphere (700 vpm) and resuspending in demineralized water to a concentration such that each incubation bottle received 5 mg C g$^{-1}$ DW peat in an added volume of 10ml. This concentration was chosen to simulate a 50% increase in local average belowground litter production (Malmer et al., 2005), a previously observed effect size in sub-arctic systems under warming treatments (Press et al., 1998). Control pots received 10 ml sterile, demineralized water at the same time as the other treatments received substrate.

Incubations were conducted in three climate chambers, with the appropriate temperatures set, at 90 % humidity and constant darkness. Peat in each bottle was mixed during the initial substrate addition and whenever peat was removed for sampling purposes. Bottles were weighed at least once every 5 days and appropriate amounts of sterile, demineralized water were added to maintain peat moisture content at a constant 80 % w/w.

Measurements as described below were taken at the following intervals: respiration measurements: Days 1*, 2, 6, 9*, 14, 19, 26*, 33, 48, 58* (where * denotes samples were also analyzed for 13C); Nitrogen pool measurements: Days 3, 7, 15, 29, 45, 60; Potential enzyme activity: Days 4, 13, 44, 60 DNA sample for microbial community structure: Days 5 and 45. In addition 8 “baseline” samples for DNA extraction were taken at Day 0, i.e. from the homogenized soil just prior to placement in the mesocosms.

### 5.3.3 Respiration measurements

Instantaneous rates of respiration were estimated by sealing the incubation bottles (which were left unsealed at all other times), sampling the headspace with double-headed needles and 12 ml exetainers that were pre-treated by flushing with N$_2$ gas and evacuated with a vacuum pump. Short-term time series consisting of three samples were taken. The time between first and last sampling was generally between 60 and 120 minutes depending on the stage of the incubation (later measurements were extended to accommodate slower respiration rates) and was based on pilot studies that showed linear accumulation of CO$_2$ without saturation over this time period. Concentration of CO$_2$ in exetainers was determined with a HP 5890A gas chromatograph (Hewlett Packard, Palo Alto, USA) with regular calibration. Calibration lines were always linear with $R^2 > 0.999$. Respiration rates were calculated from the slope of
the graphs of CO$_2$ concentration and sampling time, and converted to mg C h$^{-1}$ g$^{-1}$ DW peat, allowing for changes to both the mass of remaining peat in the incubation jars and the volume of the headspace as the experiment progressed.

On occasions where samples were also used for $^{13}$C measurements, a fourth headspace sample was taken at the final sampling moment. $^{13}$C measurements were carried out on a gas chromatograph coupled to a stable isotope ratio mass spectrometer (Gasbench II and Delta Plus ThermoFinnigan).

5.3.4 Potential Enzyme Activity measurements

Potential enzyme activity assays were conducted as described in Chapter 3 using the protocol of Steinweg and McMahon (http://enzymes.nrel.colostate.edu/). Substrates labeled with 7-amino-4-methylcoumarin (MUC) or methylumbelliferone (MUB) were selected to estimate the relative pool sizes (i.e. activity with saturating substrate concentrations) of enzymes responsible for the hydrolysis of four peptide and four carbohydrate substrates. Specifically, we used the model peptides: L-leucine-7-amido-4-MUC (henceforth, Leu), L-alanine-7-amido-4-MUC (Ala), L-lysine-alanine-7-amido-4-MUC (Lys-Ala), and L-alanine-alanine-phenylalanine-7-amido-4-MUC (AAP); and for the carbohydrate substrates: 4-MUB-B-D-xylopyranoside (Xylo), 4-MUB-β -glucopyranoside (Beta-glucose), 4-MUB - β -glucopyranoside (Alpha-glucose) and 4-MUB-β -cellobioside (Cello) (all substrates supplied by Sigma-Aldrich). The protocol includes construction of unique seven-point calibration curves for each peat sample and provides better correction for non-linear fluorescence quenching dynamics than the usual single-point correction.

We followed the protocol cited above using slurries created by homogenizing 4 g fresh weight of soil in 90 ml 0.5 M sodium acetate buffer (pH 5). Incubations were at 9 $^{\circ}$C for 24 hours. Fluorometric measurements were made on a Spectramax Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, USA) with excitation wavelength of 365 nm and emission detection at 450 nm. All measurements were converted to nanomols per gram dry weight per hour for statistical analysis.

5.3.5 Nitrogen Pool Measurements

Nitrogen pool measurements were conducted as described in Chapter 3. Briefly, on each sampling occasion, two samples of 5 g fresh weight peat were taken for nitrogen pool measurements. One sample was immediately extracted by shaking for 2 hours
in 0.5M K₂SO₄, and the resulting solution filtered and frozen before further analysis. The second sample was fumigated in chloroform for 5 days prior to an identical extraction procedure. Following extraction, a subsample of the unfumigated extract was kept aside for measurements of inorganic N (NH₄ and NO₃). The remaining unfumigated extract and fumigated extract were digested with potassium persulfate (Cabrera & Beare, 1993) to oxidize all nitrogen species to nitrate. Ammonia and nitrate concentrations were then determined for each of the three sample types and the pool sizes calculated as follows:

\[
N_{\text{inorganic}} = \text{Ammonia}_{\text{Unfumigated, undigested}} + \text{Nitrate}_{\text{Unfumigated, undigested}}
\]

\[
N_{\text{organic}} = \text{Nitrate}_{\text{Unfumigated, digested}} - N_{\text{inorganic}}
\]

\[
N_{\text{microbial}} = \frac{(\text{Nitrate}_{\text{Fumigated, digested}} - N_{\text{organic}} - N_{\text{inorganic}})}{K_{EN}}
\]

Where \( K_{EN} = 0.45 \), a correction factor to allow for incomplete solubilization of microbial biomass (Jenkinson et al., 2004). We did not empirically determine the optimal value for \( K_{EN} \), but given that all our extractions were from a single homogenized soil matrix, and we primarily use our results for comparison between treatments, any error in the choice of \( K_{EN} \) will not have biased our results.

5.3.6 Microbial Community Structure

Samples for DNA extraction (approx. 2 g fresh weight) were immediately stored at 20 °C for later extraction. After thawing, 0.5 g FW subsamples were extracted using the Mobio PowerSoil ® DNA isolation kit (Carlsbad, USA) following the standard protocol, except for the use of a Fast Prep bead-beating apparatus (Bio101, USA) during the cell lysis step. This kit had been selected as optimal in terms of yield, quality and community profile reproducibility in earlier tests. Bacterial community profiles were constructed for each sample by estimating the relative abundance of six large major bacterial taxa: Acidobacteria, Actinobacteria, Bacteroidetes, γ-proteobacteria, α-proteobacteria and β-proteobacteria. These taxa were selected on the basis of their predominance in acidic peatland soils (Dedysh et al., 2006; Wilhelm et al., 2011) and on the identification of major taxa in a preliminary cloning and sequencing analysis of DNA isolated and pooled from a representative selection of our DNA samples.
We used the phylum specific 16S rRNA gene primers and reaction conditions specified in Chapter 3 and de Boer et al. (2011). We also estimated Eubacterial 16S rRNA gene copy number with universal primers for use as a normalizing constant, thus allowing for differences in nucleic acid extraction efficiency and total sample bacterial abundance. All qPCR runs used triplicate six-point standard curves and no-template controls and duplicate samples. Samples were randomly assigned to plate runs, and a consistent internal standard was used for correction of inter-plate differences. All reactions were run on an AB 7300 Real-Time PCR System (Applied Biosystems, Branchburg, USA) and, $R^2$ values of calibration lines were always $>0.98$ and apparent qPCR efficiencies (Smith & Osborn, 2009) ranged from 90 to 101%.

5.3.7 Data analysis

The time series of respiration rates generally showed an early peak (first 3-5 days of incubation), followed by a rapid decline to a fairly constant basal rate after approximately 10 days (see Results below). These non-linear dynamics make the fitting and interpretation of a non-linear repeated-measures statistical model difficult. In addition, respiration measurements in the Low temperature treatment were biased by technical difficulties with maintaining the incubation temperature while sampling the headspace. We therefore summarized the time series for each mesocosm in the Medium and High temperature treatments only with two measures: the Maximum respiration (i.e. the highest observed respiration rate in each time series, out of 9 measurements) and Basal post-peak respiration rate (the mean of the five respiration rate measurements between days 19 and 58). These summary values provide information about the effect of the treatments on two distinct phases of the microbial activity during the incubation and were analyzed separately in two-way ANOVAs, with temperature and substrate treatments as fixed factors with two and three levels each, respectively.

Measured $\delta^{13}C$ values for each sampling occasion were plotted against the reciprocal of CO$_2$ concentration to create Keeling plots (Keeling, 1958), the intercept of which was taken as the $\delta^{13}C$ value of the respired carbon at each Time $\times$ mesocosm combination. Combining these values with the $\delta^{13}C$ from Control bottles allowed calculation of the percentage of respired carbon derived from experimentally added substrate (bacterial cells or Carex roots) by the formula:
\[ P = \frac{\delta^{13}C_M - \delta^{13}C_{Bg}}{\delta^{13}C_S - \delta^{13}C_{Bg}} \]

Where \( P \) is the proportion of respired CO\(_2\) derived from experimentally added substrate, \( \delta^{13}C_M \) is the \( \delta^{13}C \) value of the respired carbon, \( \delta^{13}C_{Bg} \) is the background \( \delta^{13}C \) value (determined from Control bottles) and \( \delta^{13}C_S \) is the \( \delta^{13}C \) value of the added substrate. This approach ignores metabolic fractionation by microbes during decomposition and respiration (which may be negligible relative to substrate preference (Santruckova et al., 2000)) and allowed us to test for temporal changes in the use of the added substrate across the different temperature and substrate treatments. To this end the times series of \( P \) for all Cells and Roots mesocosms were analyzed with two-way repeated measures ANOVA (sampling day as within-subject factor and substrate and temperature as fixed between-subject factors).

Potential enzyme activities were analyzed separately for each of the eight enzymes. Initial analysis using a factorial repeated measures ANOVA model showed statistically non-significant or otherwise low magnitude interaction effects between sampling time and substrate treatment and/or temperature treatment. So, for ease of presentation and interpretation, we converted the data to weighted averages over the entire incubation period with weights determined by the time between measurements. These weighted averages were used as response variable in a factorial two-way ANOVA model, as described above for other variables.

Nitrogen pool measurement data were analyzed with a repeated measures ANOVA, with Time, Temperature and Substrate treatment as fixed factors and a sampling date as the repeated-measures term.

Relative abundances (\% of total eubacterial 16S rRNA copy number) of each of the four taxonomic groups targeted by the qPCR assays were analyzed with a permutational MANOVA model using the adonis function of the vegan package in R (Oksanen et al., 2010). Time was a repeated measures factor and Substrate and Temperature treatment were incorporated as fixed factors.
5.4 Results

5.4.1 Respiration measurements

Respiration rates showed an initial peak in the first 2 or 3 measurement periods, after which (after 5 days of incubation) they declined to a level that remained relatively constant for the remainder of the incubation. Mean maximum respiration rates varied almost two-fold among treatments and ranged from 7.1 (all respiration data expressed as µg CO₂ g⁻¹ DW soil hour⁻¹) in Control-Medium mesocosms to 12.1 in the Cells-High temperature treatment. There was a significant effect of both Temperature and Substrate treatment on these maximum rates, but no interaction between them (all results of statistical analyses are in Table 5-1). Substrate addition resulted in higher maximum respiration rates, which were increased by 20 and 23 % by the Cells and Roots treatments, respectively (relative to Control). Also, higher temperatures resulted in a 38 % higher maximum respiration rate in the High versus the Medium temperature treatment. Mean basal respiration rates ranged from 1.46 in the Roots Medium treatment to 3.06 in the Cells High temperature treatment, with 46% increase due to Cells treatment (relative to Control) and a 34% increase due to the High temperature treatment, and no significant effect of the Roots treatment (Figure 5-2).
The proportion of respired carbon derived from added substrate to total respired CO$_2$ was highest (10-12 %) at Day 1 in mesocosms receiving the Roots addition and subsequently declined to around 5% for the remainder of the incubation (Figure 5-3). In mesocosms receiving the Cells substrate addition, the peak was at 9 day and ranged from 12 to 35% of total respired CO$_2$ before declining to 5 to 10 % (Figure 5-3). There appeared to be a temperature-related phase shift in the time series of added substrate utilization, but the exact timing of this is difficult to determine given the relatively low number of sampling occasions in our design. These patterns are confirmed by the significant three-way interaction term in the repeated measures ANOVA for this data (Table 5-1).

### 5.4.2 Nitrogen pool measurements

All measured nitrogen pools showed complex responses to the combination of treatments, which varied through time (Figure 5-4). For microbial N, Substrate and Time were significant terms in the repeated measures model (Table 5-1). The main Substrate effect was a 76% increase in the microbial N content in mesocosms receiving the Cells substrate addition relative to the Control and Roots treatments (Figure 5-4). There was a significant Temperature × Substrate interaction (Table 5-1) due to different effects of substrate treatment at different incubation temperatures although these effects were always small relative to the overall substrate effect (all coefficients <10% of overall mean of the response).
Figure 5-4: Time series of nitrogen pool sizes (top row, inorganic N; centre row, microbial-N; bottom row, organic N) over 60 day incubations at 9, 11, and 14 °C with addition of dead bacterial cells (central panel), ground plant roots (right panel) or sterile water (left panel) at day 0. Lines join mean measurements at each Time x Temperature combination. Note different Y-scales for each N pool.
The measurements of organic N showed similar dynamics; the Cells treatment increased the organic N pool sizes by an average of 83% relative to Control mesocosms. Temperature and temporal effects were significant (Table 5-1), but with effect sizes lower (approximately 7% and 25% of the overall mean response, respectively) than that seen for substrate treatments. Inorganic N was generally at low levels (often below detection) except for in the early days of the incubation in the Cells substrate treatment, and at the end of the incubation in Control mesocosms (Figure 5-4). This is reflected in the significant effects of Time, Substrate and their interaction in the repeated-measures model (Table 5-1). The largest effect was due to Substrate.

### 5.4.3 Potential Enzyme Activity measurements

Potential enzyme activities integrated over the entire incubation period were positively affected by the Cells treatment in the case of three out of the four measured peptidases (Ala-, Lys-Ala and Leu-peptidases), with effect sizes ranging from 30 to 64% increase relative to the Control treatment (Figure 5-5a). In addition, there was a positive effect of the High temperature treatment (+13% relative to Low temperature) for Leu-peptidase potential activity only. Carbon cycle enzymes showed a similar pattern: the Cells substrate treatment increased potential activities by 40, 16 and 16% relative to Control and Roots treatments for alphaglucosidase, betaglucosidase and xylase, respectively (Figure 5-5b).

### 5.4.4 Microbial Community Structure

The six taxonomic groups targeted by our qPCR assays (Bacteriodetes, Acidobacteria, Actinobacteria, α-proteobacteria, β-proteobacteria and γ-proteobacteria) covered on average 53 ± 10% (SD) of the total detected eubacterial 16S rRNA gene copy numbers in each sample. Analysis of the community composition with PERMANOVA revealed that only substrate treatment and time, and their interaction had significant effects on the relative abundances of these four groups (Table 5-1). The samples taken on Day 5 from mesocosms receiving the Cells substrate treatment were significantly different from all other Substrate × Day combinations — due to an increase in the relative abundance of β-proteobacteria, and decrease in the relative abundances of α-proteobacteria, Actinobacteria and Acidobacteria (Figure 5-6). There was no detectable effect of the Temperature treatment on the microbial community composition.
Figure 5-5: Weighted averages (integrated over time) of potential enzyme activity in peat soil mesocosms measured after 4, 13, 44 and 60 days of incubation at 9, 11, or 14 °C with addition of dead bacterial cells, ground plant roots or sterile water at day 0. Potential activities were measured by incubation with fluorogenic model substrates at 9°C for 24 hours. Different letters indicate significant differences between substrate treatments (Tukey HSD, P < 0.05). Error bars indicate S.E. of sample mean for each substrate/temperature/enzyme combination.
<table>
<thead>
<tr>
<th>Term</th>
<th>Temperature</th>
<th>Substrate</th>
<th>Sampling time</th>
<th>Temperature x Substrate</th>
<th>Temperature x Time</th>
<th>Substrate x Time</th>
<th>Temperature x Sampling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>N pools</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inorganic N</td>
<td>4.8*</td>
<td>37.4***</td>
<td>3.2**</td>
<td>1.6</td>
<td>1.6</td>
<td>3.16**</td>
<td>-</td>
</tr>
<tr>
<td>Organic N</td>
<td>4.4*</td>
<td>162.3***</td>
<td>13.7***</td>
<td>1.2</td>
<td>4.2***</td>
<td>5.8***</td>
<td>-</td>
</tr>
<tr>
<td>Microbial N</td>
<td>1.2</td>
<td>104.8***</td>
<td>2.4*</td>
<td>3.2*</td>
<td>0.7</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme pools</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>3.0</td>
<td>14.3***</td>
<td>-</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.3*</td>
<td>98.2***</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine-Alanine</td>
<td>0.04</td>
<td>4.0*</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AAP</td>
<td>1.5</td>
<td>0.7</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Betaglucosidase</td>
<td>1.2</td>
<td>3.6*</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alphaglucosidase</td>
<td>0.6</td>
<td>9.3***</td>
<td>-</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylase</td>
<td>3.3</td>
<td>4.6*</td>
<td>-</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cellulase</td>
<td>0.7</td>
<td>0.8</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Microbial community structure</td>
<td>1.9</td>
<td>6.5***</td>
<td>3.7**</td>
<td>0.8</td>
<td>2.0</td>
<td>3.4**</td>
<td>-</td>
</tr>
</tbody>
</table>

*Significance levels of F-values: * p < 0.05, ** p < 0.01, *** p < 0.001. See main text for more details of statistical procedures.

**Table 5-1. Summary of F-values and significance levels of terms in (repeated measures-) ANOVA or PERMANOVA models.**
Figure 5-6: Non-metric multi-dimensional scaling plot of microbial community structure in samples taken after 5 (solid lines) and 45 (dashed lines) days of incubation at 9 or 14 °C with addition of dead bacterial cells, ground plant roots or sterile water at day 0. Community composition was determined with taxon-specific qPCR for Acidobacteria, Actinobacteria, β-proteobacteria, α-proteobacteria, γ-proteobacteria and Bacteroidetes. Arrows to the upper right indicate mapping of bacterial group relative abundances to the NMDS space, relative to the black circle (origin). X symbols represent “baseline” (Day 0) samples taken prior to the beginning of incubations.

5.5 Discussion

Using a realistic, controlled mesocosm experiment, we showed that indirect effects of temperature-driven substrate inputs dominate over direct temperature effects in controlling important components of peatland soil C and N cycling. Moreover, our results suggest that the dynamics of the microbial inputs are more important than changes to plant-derived substrate input in driving this indirect effect. We propose
that this heretofore little-studied aspect of soil C and N cycling may be responsible for the high apparent temperature sensitivity of peatland processes, and should be accounted for when developing mechanistic models of soil organic matter decomposition for this globally important and climate change-vulnerable C pool.

5.5.1 Microbe-related indirect effects dominate over direct effects of temperature

With the exception of basal and peak respiration rates (see below), the soil chemical and biological properties we measured were most strongly affected by the Cells treatment (Table 5-1), with comparatively small or non-significant effects of both temperature (increase of up to 5°C) and the addition of root-derived plant litter. The fact that microbial, organic and inorganic nitrogen pool sizes only increased in response to the Cells treatment is not surprising, given the much lower C:N ratio (6:1) of this substrate relative to either the Roots treatment (80:1), or the Sphagnum litter that makes up the majority of the organic matter in our mesocosms (87:1 for Sphagnum balticum at Stordalen; Aerts et al. 2001; 50:1-90:1, (Kuhry & Vitt, 1996)). This is evidence that nitrogen cycling in these peatland soils is dominated by tightly controlled turnover of organic N (Schimel & Bennett, 2004; Nannipieri & Eldor, 2009), and that mineralization is only detectable when large amount of relatively labile, N-rich substrate is added, making N transiently non-limiting. The fact that temperature increases alone did not increase the sizes of the available N pools contrasts with the strong (direct and indirect) effects of experimental warming observed under field conditions (Chapter 3; Dorrepaal et al., 2009), and supports the interpretation that observed temperature effects in the field are more strongly related to indirect, than to direct mechanisms.

Enzyme potential activity, and therefore levels of extracellular enzyme production by the microbial community, also showed the strongest response to the addition of the N-rich Cells substrate. This can be taken as evidence that enzyme production is itself N-limited in this system, or alternatively, that the chemical composition of the Cells substrate addition were more effective than those in the Roots substrate at inducing extracellular enzyme production (Allison & Vitousek, 2005). Potential enzyme activities under field conditions have previously been found to be insensitive to small temperature increases (Fenner et al., 2007a; Bell et al., 2010; Bell & Henry, 2011) and are more likely determined by seasonal dynamics of microbial nutrient demand, as well as levels of available inorganic nitrogen (Nemergut et al., 2008; Enowashu et al., 2009; Bell et al., 2010; Currey et al., 2010). Our experiments,
which directly controlled temperature and substrate conditions, support this common finding of non-significant effects of temperature and significant effects of nutrient supply on potential enzyme activities.

Our experimental manipulations also led to changes in the structure of the peat microbial community. A transient peak in $\beta$-proteobacteria relative abundance was observed in mesocosms in the Cells treatment at day 5, coinciding with lower abundances of $\alpha$-proteo-, Actino- and Acidobacteria. There was no significant effect of incubation temperature on microbial community composition. This shift in community composition coincided with high rates of respiration and ammonia in these samples, and supports the previously suggested idea that members of $\beta$-proteobacteria class are associated with the rapid use of labile substrates, and that Acidobacteria and Actinobacteria are associated with metabolism of more recalcitrant substrates (Fierer et al., 2007). Although our use of only three sampling points does not allow us to estimate the exact duration of the shift in community composition, we interpret this observation as evidence that microbial community shifts in response to changes in nutrient status can be rapid and transient (Bardgett et al., 2005). This implies that high temporal resolution in field community sampling is required to successfully characterize the microbial community active during the “hot moments” when critical biogeochemical processes are occurring (McClain et al., 2003).

Mesocosm respiration rate was the only measure to be altered by the temperature treatment to a comparable degree as the effects of substrate treatment (Table 5-1). Both initial peak respiration rates and steady-state basal respiration rates were positively related to incubation temperature. Soil microbial respiration has a long-established dependence on soil temperature (Lloyd & Taylor, 1994), due to both the increased rate of enzymatic reactions and the higher maintenance cost (i.e. lower growth efficiency) of the microbial biomass under higher temperatures (Steinweg et al., 2008; Allison et al., 2010b) (but see Dijkstra et al., 2011). Moreover, a positive relationship between warming and CO2 flux has been often observed in peatlands (e.g. Updegraff et al., 2001; Dorrepaal et al., 2009; Flanagan & Syed, 2011). The direct temperature effect in our study (calculated from 11 to 14 °C) was equivalent to $Q_{10}$ values of 2.9 and 2.7 for peak and basal respiration, respectively. This is considerably less than the $Q_{10}$ values calculated from previous peat soil lab incubations (Chapman & Thurlow, 1998), and lower than the apparent temperature sensitivity of peatland respiration under experimental warming at our study site ($Q_{10} >8$; Dorrepaal et al., 2009). Substrate additions also increased respiration rates. Initial peak rates were stimulated by both substrate treatments and temperature, whereas basal respiration rates later in the incubation were increased only by temperature and the Cells treatment (Figure 5-2). The effect of the Roots substrate treatment on respir-
ation was therefore transient and most likely caused by the rapid utilization of the labile fraction of the root litter, leaving relatively recalcitrant material that was not preferentially respired relative to the native SOM. Conversely, the positive effect of the Cells treatment persisted throughout the incubation, reflecting the sustained positive effect of this treatment on the total microbial biomass and enzyme production (Figure 5-3). Moreover, this positive effect of the Cells treatment was additive to that of temperature, resulting in a doubling of respiration rates, as observed previously in the field (Dorrepaal et al., 2009) and translates to a $Q_{10}$ value of 9.2. Such a high apparent temperature sensitivity when substrate inputs are included alongside temperature lends support to our main interpretation of the central importance of substrate-driven indirect temperature effects.

In total, our results show that the addition of relatively labile, high-N microbial biomass had a stronger effect on components of peat C and N cycles than a 5 °C increase in incubation temperature or the addition of plant-derived substrate. If we take the substrate treatment as a realistic simulation of temperature related increases in microbial biomass turnover (see discussion of the treatment realism below) then our conclusion is that this indirect effect of warming is more important than direct temperature effects on soil C and N processes. The idea that microbial dynamics can have strong consequences for biogeochemical fluxes is not new (Zak et al., 1990; Schimel et al., 2007; Schmidt et al., 2007), but this had yet to quantified. The novel contribution of our study is the explicit, controlled test of the relative effects of this mechanism versus increased temperature alone. This study therefore suggests an empirically supported, and biologically plausible interpretation, of high apparent temperature sensitivity of peat soil processes observed in the field and lab (Chapter 3; Dorrepaal et al., 2009), with the potential to be generalized to soil processes in other ecosystems (see below).

### 5.5.2 Realism of the experimental system

The utility of using our mesocosm results for interpreting field measurements depends on the realism of our model system. Table 5-2 compares the range of values obtained for a subset of variables measured in our experiment with values obtained in our previous field measurements and previously published values in subarctic peatlands, both nearby the source of peat material for the current experiment (Chapter 3; Dorrepaal et al., 2009) and, for the sake of comparison, a similar system in Canada (Pelletier et al., 2011). In general, there is a good coincidence between the measurements from our mesocosms and those from previous field measurements. The only
Table 5-2: Comparison of selected measures from the present study with previous measurements made either directly in the field (Dorrepaal et al., 2009; Pelletier et al., 2011) or from samples collected from field experiment (Chapters 3 & 4).

<table>
<thead>
<tr>
<th>Measure</th>
<th>Current experiment</th>
<th>Published/previous</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.26 -3.83 CO₂ m⁻² day⁻¹</td>
<td>2.4 -16.8 CO₂ m⁻² day⁻¹</td>
<td>Dorrepaal et al. (2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.06 -3.9 CO₂ m⁻² day⁻¹</td>
<td>Pelletier et al. (2011)</td>
</tr>
<tr>
<td>Microbial N</td>
<td>0.16 - 1.04 mg N g⁻¹ DW</td>
<td>0.29- 0.83 mg N g⁻¹ DW</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Organic N</td>
<td>&lt; 0.001 - 0.059 mg N g⁻¹ DW</td>
<td>0.03 - 0.32 mg N g⁻¹ DW</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Ammonia</td>
<td>&lt; 0.001 - 0.10 mg N g⁻¹ DW</td>
<td>0.002 - 0.026 mg N g⁻¹ DW</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>% β-proteobacteria</td>
<td>0.12 - 1.7%</td>
<td>0.4 - 3.1%</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>% Acidobacteria</td>
<td>14.4 - 44.3%</td>
<td>16.8 - 60.0%</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Leucine-peptidase</td>
<td>5 - 12 nmol g⁻¹ DW h⁻¹ at 9 °C</td>
<td>5 - 39 nmol g⁻¹ DW h⁻¹ at 4 °C</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Beta-glucosidase</td>
<td>27 - 558 nmol g⁻¹ DW h⁻¹ at 9 °C</td>
<td>51 - 650 nmol g⁻¹ DW h⁻¹ at 20 °C</td>
<td>Chapter 4</td>
</tr>
</tbody>
</table>

A measurement for which there is an order of magnitude discrepancy is the maximum ammonia measurement, which is higher in our mesocosms compared to field measurements (0.10 and 0.026 mg N g⁻¹ DW respectively). This is attributable to a single (perhaps outlier) ammonia measurement in the Cells treatment at day 5 (Figure 5-3). Thus, although highly artificial in terms of the removal of vegetation influences, constant temperature and peat moisture, we conclude that our experimental set-up gave a reasonably good approximation to field conditions, at least as regards the measured within-soil processes.

We are also confident that our choice of substrate addition and temperature treatments represent realistic simulations of field conditions. The Cells treatment consisted of adding microbial organic matter with a N content equivalent to 50 % of the peak microbial biomass N under warming in these peatlands (Chapter 3). Our hypothesis concerns warming-induced increases in the early season flush of labile carbon and nutrients from the over-wintering microbial biomass (Zak et al., 1990; Deluca et al., 1992; Larsen et al., 2007). Our imposed treatment is on the same order as observed organic N accumulations in our previous study and, as such, is within a reasonable range to test this hypothesis. Our Roots addition treatment was designed to simulate a warming-induced increase in the below-ground litter production of vascular plants under warming. Studies in another high-latitude peat bog located 9 km west of our field site have found that experimental warming of 1 to 2 °C increases the litter pro-
duction of the vegetation by around 50 % (Press et al., 1998), and our treatment was
designed to mimic this, based on estimates of belowground litter production in the
same mire (Stordalen) as where our peat material was collected (Malmer et al., 2005).
Finally, our choice of control incubation temperature was based on typical summer
soil temperatures at 10 cm depth in Northern Sweden (unpublished data, VU Am-
sterdam). We used the 11 °C treatment to simulate the typical warming effect of
ecosystem climate manipulations (Marion et al., 1997), and the 14 °C treatment to
simulate long-term (≈ 100 year) predicted temperature increases in the sub-arctic
(IPCC, 2007). In summary, in our mesocosm experiment, although artificial, we
have used a set of treatments that simulated a realistic range of field conditions and
scenarios, and these peat mesocosms are comparable to the field situation for a range
of measured parameters.

5.5.3 Microbial dynamics and apparent temperature sensitivity: a neg-
lected piece of the puzzle?

The mechanisms underlying the temperature sensitivity of soil organic matter decom-
position are still a matter of debate despite decades of experimental and modeling
studies. A large part of this uncertainty results from the difficulty of distinguishing
the intrinsic temperature sensitivity of a given process from the apparent sensitiv-
ity realized under natural conditions under multiple constraints. Following from this
are the difficulties inherent in scaling-up results from short-term incubation experi-
ments to realistic, longer-term C balances (Kirschbaum, 1995; Davidson & Janssens,
2006; Davidson et al., 2006; Kirschbaum, 2006; Subke & Bahn, 2010; Conant et
al., 2011). Much of the recent discussion has focused on the relative temperature
sensitivity of the degradation of different types of soil organic matter (Giardina &
Ryan, 2000; Conant et al., 2008; Craine et al., 2010). Other authors have suggested
a possible role of substrate supply in driving apparent temperature sensitivities, and
focus either on the seasonality of inputs of plant-derived substrate (Gu et al., 2004;
Kirschbaum, 2006), or on micro-scale effects of temperature and moisture on sub-
strate supply via diffusion limitation (Davidson et al., 2011). Moving beyond this
focus on the quality and supply rate of SOM, two recent reviews have also pointed to
the importance of accounting for the size, composition and physiological status of the
soil microbial biomass in explaining the temperature sensitivity of SOM decomposi-
tion (von Lützow & Kögel-Knabner, 2009; Conant et al., 2011). Our present finding
that microbe-derived substrate input has stronger effects than temperature and plant-
derived substrate on peat C and N cycle processes, along with the field observations of
a warming effect on the seasonal dynamics of the microbial biomass and soil N pools

94
(Chapter 3), provide empirical, quantitative support for these suggestions.

There is an extensive body of conceptual and empirical literature describing the seasonal dynamics of soil microbial biomass and the potential implications for biogeochemical fluxes (e.g., Brooks et al., 1998; Wardle, 1998; Schmidt et al., 2007; Jeffries et al., 2010). We propose that this knowledge can be used to constrain models that explicitly include microbes and their response to temperature (both in terms of biomass dynamics and enzyme production) to explore the temperature sensitivity of soil respiration and decomposition (von Lützow & Kögel-Knabner, 2009). Several microbe-explicit models that incorporate enzyme dynamics already exist (e.g., Schimel & Weintraub, 2003; Moorhead & Sinsabaugh, 2006), and they are actively being developed to include microbial biomass dynamics and analyze the potential responses of soil carbon dynamics to climate change (Allison et al., 2010b; Todd-Brown et al., 2012). It remains to be seen how the inclusion of microbial biomass dynamics in models of soil C and N cycling will effect predictions of future dynamics at local to global scales. What is clear, however, is that such a synthesis is necessary to fully understand climate-carbon cycle feedbacks (Bardgett et al., 2008) and to increase our ability to make robust and well-informed predictions about the future of the Earth system.

### 5.6 Acknowledgements

We thank E. Tóth, B. Bruning, F. Keuper and J. van Hal for assistance with labwork, and J. Kamstra and P. Cenijn for access to a fluorometer. W. Rling generously provided facilities for molecular work. This study was financially supported by the Netherlands Organisation for Scientific Research (NWO) by NWO-ALW grant 816.01.012 to RA and GAK. We thank Abisko Naturvetenskapliga Station for hosting us during fieldwork and especially B. Wanhatalo for valuable assistance with sample collection.