# Biogeochemical processes in frozen soils

## Unfrozen water in frozen soils and factors regulating carbon mineralization at low temperatures

Stina Harrysson Drotz

*Faculty of Forest Sciences*
*Department of Forest Ecology and Management*
*Umeå*

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## Abstract

High latitude ecosystems are important for global carbon (C) balances and are among the most sensitive ecosystems to climate change. Microbial $CO_2$ production in soil is known to proceed at temperatures < 0°C in these regions and winter $CO_2$ emissions can significantly affect annual C balances. However, the low-temperature processes involved were poorly understood. In frozen soils, the microbial activity must be confined to small pools of liquid water present in the bulk soil. Therefore, in laboratory incubation and NMR experiments I have investigated: the distribution of unfrozen water in frozen boreal forest soils; its implications for the mineralization of soil organic matter (SOM) and microbial substrate utilization under frozen conditions; factors controlling these phenomena and their temperature responses. The results show that the osmotic potential of unfrozen water contributes 20-69% of the total water potential in frozen soil, in contrast to unfrozen soil where its contribution is generally negligible. They also show that recalcitrant SOM components, such as aromatics and alkyl C, have positive effects on unfrozen water contents, and thus on $CO_2$ production in frozen soils. Further, temperature responses of $CO_2$ production in frozen soils are controlled by unfrozen water, and estimated biochemical $Q_{10}$-values were consistent with thermodynamic theory after factoring out the effects of water availability. In addition, both catabolic and anabolic processes can proceed at -4°C and no clear differences in C allocation patterns of metabolised substrates were observed across the -4°C to +9°C temperature range. However, at < 0°C the soil microbes required longer times to adapt and utilise substrates maximally than at > 0°C. At -4°C, this adaptation was associated with increased cell membrane fluidity, and resulted in significant increases in $CO_2$ production. The findings contribute to the general understanding of low temperature microbial processes; indicating that the hierarchy of controlling factors changes as soil freezes, but microbial metabolism is similar in frozen and unfrozen soil. The results have important implications for the conceptualization of processes related to soil C dynamics.



*Keywords:* frozen soil, unfrozen water, NMR, water potential, soil organic matter, $CO_2$, carbon, temperature response, boreal, catabolism, anabolism, microbial activity, C mineralization.



*Author's address:* Stina Harrysson Drotz, SLU, Department of Forest Ecology & Management, SLU, SE-901 83 Umeå, Sweden
*E-mail:* stina.harrysson@sek.slu.se

# Contents

# List of Publications

This thesis is based on the work described in the following papers, referred to by the corresponding Roman numerals in the text:

I **Harrysson Drotz S**., Tilston E. L., Sparrman T., Schleucher J., Nilsson M., and Oquist M. G. (2009) Contributions of matric and osmotic potentials to the unfrozen water content of frozen soils. *Geoderma* 148(3-4), 392-398.

II **Harrysson Drotz S**., Sparrman T., Schleucher J., Nilsson M., and Oquist M. G. (2010) Effects of soil organic matter composition on unfrozen water content and heterotrophic $CO_2$ production of frozen soils. *Geochimica et Cosmochimica Acta* 74(8), 2281-2290.

III Oquist, M.G., Sparrman, T., Klemedtsson, L., **Harrysson Drotz, S**., Grip, H., Schleucher, J., Nilsson, M. (2009). Water availability controls microbial temperature responses in frozen soil $CO_2$ production. *Global Change Biology* 15(11), 2715-2722.

IV **Harrysson Drotz S**., Sparrman T., Schleucher J., Nilsson M., and Oquist M. G. Microbial activity in frozen boreal forest soils – potentials for catabolic and anabolic processes under frozen conditions. *Manuscript*.

# 1 Introduction

## 1.1 Background

The organic soil carbon (C) pool in the top metre of the entire land area of the earth amounts to ca. 1500 Pg, while the atmosphere contains around 750 Pg of C (Jobbagy & Jackson, 2000; Batjes, 1996). The boreal forest covers a large part of the northern hemisphere (Figure 1) and about 40 % of the soil C pool is stored in high latitude ecosystems (IPCC, 2000). Through mineralization of soil organic matter (SOM), the stored C is released back to the atmosphere as $CO_2$, hence these ecosystems are very important for global C balances. Most investigations aimed at quantifying the C source/sink relationship of high latitude ecosystems have been carried out during the growing season, when the vegetation is fixing C through photosynthesis and the soil temperature is high. However, the importance of the winter season for the C balance is becoming increasingly recognized, and it is evident that a significant proportion of the C fixed by the system during summer may be lost during the following winter through degradation of SOM via heterotrophic microbial activity (Monson *et al.*, 2006; Oechel *et al.*, 2000). Microbial activity can occur even if the soil temperature is < 0°C and soils are frozen (Panikov *et al.*, 2006; Oquist *et al.*, 2004; Price & Sowers, 2004; Schadt *et al.*, 2003; Brooks *et al.*, 1997). Thus, it is becoming increasingly evident that microbial processes in the soils of high latitude ecosystems during the winter make very important contributions to atmospheric sink/source dynamics of greenhouse gases (Oechel *et al.*, 2000; Oechel *et al.*, 1997; Zimov *et al.*, 1996) and (hence) phenomena linked to radiative forcing and global climatic change. However, our understanding of the environmental factors regulating soil microbial activity in cold and frozen soils is poor.

It has been suggested that soil microorganisms have limited capacity/ability to utilize substrates at sub-zero temperatures (Nedwell, 1999) and that the soil microbial population shifts from growth to survival metabolism when subjected to freezing (Schimel *et al.*, 2007). Soil microbial catabolic processes (and concomitant $CO_2$ production) are known to occur in frozen soils because biogenic $CO_2$ production has been detected in them (Panikov *et al.*, 2006; Schimel & Mikan, 2005; Mikan *et al.*, 2002). However, there is still little knowledge of the extent to which microbial growth-sustaining anabolic processes can occur in them.

If the soil microbial community is to remain metabolically active as temperatures drop below 0°C and soil freezes, microorganisms must overcome quite severe changes in their physicochemical environment. Notably, when soil freezes there are dramatic reductions in the amount of liquid water (Romanovsky & Osterkamp, 2000), accompanied by putative reductions in the water films on particle surfaces and the osmotic potential of the remaining unfrozen water (Torrance & Schellekens, 2006). The availability of unfrozen water is believed to be a key determinant of microbial activity at sub-zero temperatures (Price & Sowers, 2004), and hence microbial $CO_2$ production from frozen soils, since important processes such as nutrient diffusion cannot occur in frozen water. Coefficients (e.g. $Q_{10}$-values; the factors by which rates of processes increase with a 10°C increase in temperature) of temperature responses of soil $CO_2$ production or emission reported for frozen systems are often several orders of magnitude higher than those of unfrozen systems (Monson *et al.*, 2006; Elberling & Brandt, 2003; Mikan *et al.*, 2002). However, it has been suggested that such high $Q_{10}$-values do not represent the direct biochemical response and are believed to emanate from other factors affecting the temperature response, such as water availability, substrate availability and substrate utilization (Davidson & Janssens, 2006; Davidson *et al.*, 2006).

In this thesis I have investigated the factors controlling the unfrozen water content of surface layers of boreal forest soils and the implications of this unfrozen water for the mineralization of SOM and microbial activity in the frozen soil matrix. In order to gain a deeper understanding of microbial life in frozen soils I also studied changes induced by freezing in the soil microbial population's affinity for and utilization of substrates.

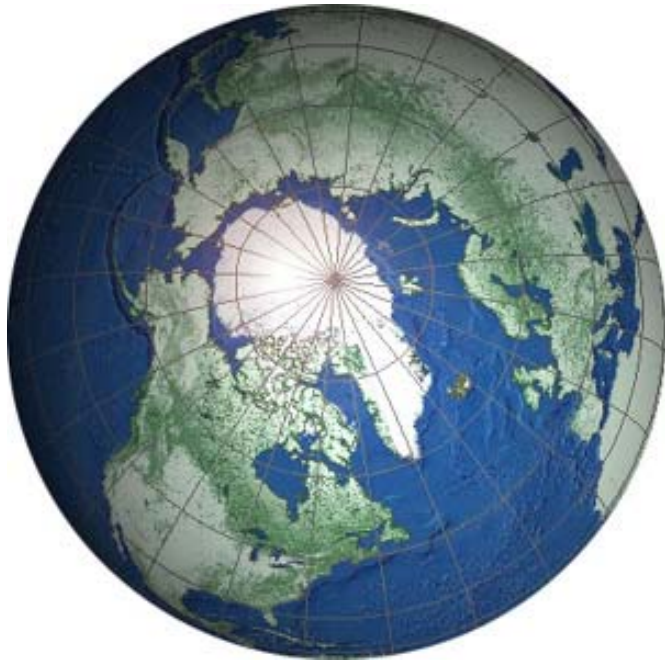


*Figure 1.* Illustration of the boreal forest region of the northern part of the earth. Increases in the darkness of the green coloration indicate increases in the percentage tree cover. The figure is from the MODIS Tree Cover data, taken from (*http://www.whrc.org/borealNAmerica/index.htm*), displaying the forests of North America and Eurasia.

## 1.2 Objectives

The main objective of this project was to identify and quantify environmental controls on biogeochemical processes of frozen boreal forest soils. The work included investigations of factors regulating the unfrozen water contents of frozen soils, the importance of liquid water for the microbial activity in frozen soils, and substrate utilization by soil microbes under frozen conditions. The thesis is based on studies reported in the four appended papers (Papers I, II, III and IV).

In Paper I, we wanted to elucidate the factors that regulate the unfrozen water contents of frozen soils, since unfrozen water was believed to be important for their C mineralization rates. The aims were: i) to determine the extent to which the unfrozen water content in frozen bulk soil is determined by particle size (matric potential) and soil water solutes (osmotic

potential), and ii) to determine the contributions of matric and osmotic potentials to the total water potential of unfrozen water in frozen boreal forest soils. Since it has been suggested that solutes in the water phase are concentrated as the soil freezes (Torrance & Schellekens, 2006), we hypothesized that both the matric and osmotic potentials contributed to the total water potential of frozen soils.

The aim of Paper II was to evaluate the effects of SOM composition on the amount of unfrozen water, the pore sizes where unfrozen water resides, and microbial $CO_2$ production in frozen boreal forest soils. The chemical composition of SOM was characterized by Cross Polarization-Magic Angle Spinning (CP-MAS) NMR spectroscopy. We hypothesized that the SOM structure and composition could determine the capacity of frozen soils to retain unfrozen water, which in turn is a major determinant of heterotrophic $CO_2$ production under frozen conditions.

In Paper III, we investigated the influence of unfrozen water content in frozen soil on the observed temperature responses in biogenic $CO_2$ production across the freezing point. This was done by combining $^{2}H$ NMR measurements of unfrozen water contents of frozen soil samples with measurements of biogenic $CO_2$ production. We hypothesized that the amount of available water at sub-zero temperatures has a major impact on the observed temperature responses of $CO_2$ production, due to the influence of unfrozen water on biogenic $CO_2$ production in frozen soil.

The aim of Paper IV was to investigate substrate utilisation of the soil microbial population and possible changes in the allocation of metabolized C between $CO_2$ production and cell constituents as soils change from an unfrozen to a frozen state. This was done by evaluating the microbial turnover of $^{13}C$-labelled glucose in the organic surface layer of frozen boreal forest soils at various temperatures using $^{13}C$ MAS NMR spectroscopy and $^{13}CO_2$ respiration measurements. We hypothesized that both anabolic and catabolic processes could proceed under frozen conditions, although metabolic rates would be lower in frozen soils.

## 1.3 Microbial metabolism and soil organic matter (SOM) degradation

The heterotrophic microbial community of the soil degrades the SOM in order to obtain energy for respiration and cell growth. Hence, $CO_2$ production is a result of the degradation of SOM and the oxidation of carbohydrates can be generalized as,

$$CH_2O + O_2 \rightarrow CO_2 + H_2O + \text{intermediates} + \text{cellular material} + \text{energy} \quad \textit{(Eq. 1)}$$

Soil microbial activity and the associated $CO_2$ production are affected by numerous environmental factors, but the main factors are the availability of water, electron donors and acceptors (substrate quality and abundance), and temperature (Davidson & Janssens, 2006; Davidson *et al.*, 2006; Schimel & Mikan, 2005; Mikan *et al.*, 2002). The effect of substrate quality on respiration is linked to the biochemical pathways involved and the associated activation energies, which are reflected in the energy required to degrade the substrate (Bosatta & Ågren, 1999), while substrate availability is affected by diffusion rates, and hence soil water content. For instance, when conditions are dry, and soil water contents low, heterotrophic activity may be limited by the supply of substrates due to the low rates of diffusion (Davidson & Janssens, 2006). Generally, when the temperature increases the rate of organic matter degradation increases. The effect of temperature on biochemical response in $CO_2$ production is often described by the factor called $Q_{10}$. For unfrozen systems not subjected to e.g. water stress, $Q_{10}$ values around 2-3 have been reported (e.g. Elberling & Brandt, 2003).

Microbial metabolism involves both catabolic processes (including respiration and fermentation, in which metabolic co-factors, e.g. NADH, are regenerated) and anabolic processes in which cell constituents (e.g. proteins and phospholipids) are synthesized and incorporated into biomass (Gottschalk, 1985). For cell membranes to be physiologically active the acyl chains of their phospholipids need to be in a liquid-like state to keep the membrane proteins active (Box 1). Both bacteria and fungi are capable of adjusting their cell membrane composition in response to temperature changes, thereby maintaining their membranes in a lamellar liquid crystalline phase (Rilfors & Lindblom, 2002).

**Box 1. Phospholipids in cell membranes**

In aqueous environments the phospholipids of the cell membrane (e.g. phosphatidylcholine) associate in a double-layered structure (Gottschalk, 1985). The cell membranes of bacteria contain various phospholipids, e.g. phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. The structure of the phospholipid dioleoylphosphatidylcholine (DOPC) is illustrated in Figure 2. The fluidity of the cell membrane is determined by the degree of unsaturation of the lipid acyl chains, which is determined by the number of *cis* double bonds. When *cis* double bonds are incorporated into the fatty acid chains of a phospholipid, a bend in the chain is induced (Mathews *et al.*, 2000). This increases the fluidity of the membrane by inhibiting close packing of the phospholipids and allows the membrane to remain flexible at lower temperatures (Mathews *et al.*, 2000).

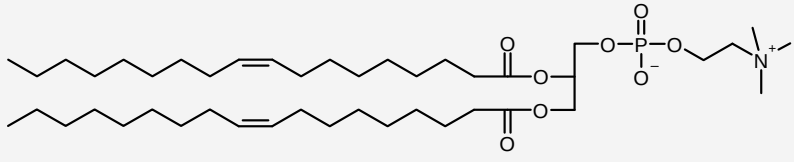


*Figure 2.* Chemical structure of dioleoylphosphatidylcholine (DOPC).

## 1.4 Catabolic and anabolic processes in frozen soil

It is known that soil microbial $CO_2$ production (catabolism) occurs in frozen soils (Panikov *et al.*, 2006; Schadt *et al.*, 2003; Mikan *et al.*, 2002; Brooks *et al.*, 1997). Indeed, microorganisms in tundra soil samples have been found to produce $CO_2$ under extremely cold conditions (down to -39°C) (Panikov *et al.*, 2006), and it has been suggested that there is no evidence of a minimum temperature for metabolism (Price & Sowers, 2004). However, knowledge about anabolic processes is poorer. It has been suggested that the soil microbial community may have problems sequestering substrates at temperatures around and below 0°C (Nedwell, 1999) and that microbes may alter their allocation of resources from growth to survival-related metabolism when the soil is subjected to stresses, such as freezing (Schimel *et al.*, 2007). It has also been suggested that microorganisms make adjustments at the molecular level (e.g. in amino acid contents and protein expression) in response to temperature reductions (Deming, 2002) that enable them to survive and remain active in cold conditions. Thus, since liquid water can exist in frozen soil and

(furthermore) substrates are believed to be concentrated as the soil freezes (Torrance & Schellekens, 2006; Sparrman *et al.*, 2004), it should theoretically be possible for both catabolic and anabolic microbial processes to continue at sub-zero temperatures, providing that microorganisms can tolerate the low soil water potentials required to keep water in its liquid state and maintain their membrane functions.

For frozen systems, $Q_{10}$-values up to several orders of magnitude higher than for unfrozen systems have been reported (Monson *et al.*, 2006; Elberling & Brandt, 2003). However, these high $Q_{10}$-values are unrealistic, because they do not correspond to biophysically reasonable activation energies for microbial aerobic degradation. It has been suggested that $Q_{10}$-values exceeding 2.5 emanate from other factors affecting the estimated temperature response, e.g. limited substrate diffusion caused by thin soil water films (Davidson *et al.*, 2006). The factors affecting temperature responses have also been suggested to be linked to phenomena such as shifts in species composition and/or substrate use (Schimel & Mikan, 2005; Schadt *et al.*, 2003). However, liquid water is essential for all biological life and water availability is drastically reduced as the soil freezes (Sparrman *et al.*, 2004; Romanovsky & Osterkamp, 2000). Thus, unfrozen water in frozen soil is expected to influence diffusion rates and microbial activity in a similar fashion to that described for soil in dry conditions (Davidson & Janssens, 2006; Davidson *et al.*, 2006).

## 1.5 Unfrozen water in frozen soil

When soils transform from an unfrozen to a frozen state the availability of liquid water is reduced, with accompanying affects on substrate availability, hence unfrozen water becomes a key factor affecting the potential rates of microbial C mineralization in frozen soils. The ability of a soil to retain unfrozen water is controlled by its water potential, which regulates the freezing point of the bulk soil water.

### 1.5.1 Water potential

The total water potential ($\Psi_{tot}$) controls the unfrozen water content in frozen soil by determining the freezing point of the soil solution. Thus, in contrast to unfrozen conditions the temperature directly affects the water potential in the frozen soil matrix. Water potential is a concept that describes the availability of water for e.g. microorganisms. For example, sea water has a lower water potential than freshwater, hence water molecules crystallize less readily in it, so saltwater has a lower freezing point than

freshwater. At a given temperature < 0 °C the water potential must exceed specific values for water to remain liquid (Atkins, 1990). The total water potential can be described by the van't Hoff relationship for an ideal solution, in which the matric potential is zero and total water potential is equal to the osmotic potential (Atkins, 1990):

$$\Psi_{TOT} = R \cdot T \cdot M \qquad \textit{(Eq. 2)}$$

where the gas constant R equals 0.008315 L· MPa· $K^{-1}$· $mol^{-1}$, T is the temperature (K), and M is the molar concentration of the solute (mol· $L^{-1}$).

Eq. (2) gives absolute values of total water potential, but the water potentials are expressed as negative values in this thesis because they represent the 'suction potential' of soil. Thus the soil water potential has two components: the matric potential ($\Psi_M$) and the osmotic potential ($\Psi_O$). Boreal forest soils have high SOM content and both the content and the composition of SOM influence the soil water potential via effects on the osmotic and matric potential (Hillel, 2005). The matric potential is governed by several interrelated factors, *inter alia* the pore size distribution, the distribution and characteristics of particle surfaces, and both the quality and quantity of soil organic matter (Hillel, 2005; Hohmann, 1997; Andersson & Wiklert, 1972). The osmotic potential of the soil water is affected by the concentration of solutes, which decrease the water potential and depress the freezing point of solutions (Atkins, 1990).

The importance of osmotic potential for the water potential in frozen soil has been discussed in previous studies (e.g. Torrance & Schellekens, 2006), but there have been few attempts to quantify it. It has been suggested that the osmotic potential of unfrozen water in frozen clay and silt soils is negligible (Suzuki, 2004). However, if the amount of unfrozen water was regulated solely by the matric potential (via capillary forces), the pores in the soil (Hillel, 2005) containing unfrozen water would theoretically be smaller than most soil microorganisms (Christensen *et al.*, 1995; Bakken & Olsen, 1987). This strongly conflicts with observations of microbial activity in frozen soils (down to -39°C, Panikov *et al.*, 2006). For instance, if only matric potential governed the entire freezing point depression at -40°C, liquid water would only be present in pores with diameters ≤ ca. 7 nm; approaching molecular dimensions and hence far too small to contain any known soil microorganisms (Christensen *et al.*, 1995; Bakken & Olsen, 1987). This pore size estimate is based on the assumption of spherical pores, and thus does not reflect the true situation in natural

soils, but it still provides an indication of the maximum sizes of pores with unfrozen water (or the thickness of water films) in frozen conditions. However, if osmotic potential also contributes to the total water potential of frozen soil, liquid water could be present in larger pores, enabling microbial activity to occur even at extremely low soil temperatures.

### 1.5.2 Effect of SOM on soil water-holding properties

SOM strongly influences the liquid water contents of soils by effects on both the matric and osmotic potential (Hillel, 2005; Andersson & Wiklert, 1972). SOM contains complex mixtures of substances, with diverse chemical groups, and its soil-water interactions depend on both the nature of its constituents and surface properties. Chemical groups typically found in SOM can be roughly divided into alkyl C (constituents, *inter alia*, of waxes or lipids), O-alkyl C (e.g. carbohydrates), aromatic and O-aromatic C (constituents, *inter alia* of lignin and tannins), and carbonyl C (e.g. ketone and aldehyde groups) (Nelson & Baldock, 2005; Kögel-Knabner, 2000; Preston *et al.*, 1994a). Some of these constituents degrade much more readily than others. Thus, when SOM degrades, the proportions of recalcitrant SOM compounds increase (Berg & Meentemeyer, 2002; Preston *et al.*, 2000; Preston, 1996). In addition, the density of the soil increases, resulting in a shift in pore size distribution with a concomitant increase in the relative proportion of smaller pores (Päivänen, 1973). The proportion of pores in which unfrozen water can reside depends on the pore size distribution of the soil. Thus, SOM composition can be an important determinant of the amount of unfrozen water that can be retained under frozen conditions.

## 1.6 NMR spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is traditionally mainly used in organic chemistry, biophysics and medicine. However, it also has many potential ecological, soil science, and biogeochemical applications. Its greatest advantages are the possibility it provides to identify different compounds in samples, and to examine samples at a detailed structural level, often non-invasively. Both solid state $^{13}C$ NMR spectroscopy and solution techniques have been used for several applications in soil investigations, such as structural characterization of SOM and plant litter, and studies of substrate turnover in soil (Nelson & Baldock, 2005; Kögel-Knabner, 2002; Lundberg *et al.*, 2001; Preston, 2001; Preston *et al.*, 2000; Wilson, 1987).

The fundamental basis of NMR is that the nuclei of many isotopes possess a quantum-mechanical property called spin, which gives them a magnetic moment. Therefore, atoms containing such nuclei adopt two or more energy levels when placed in an external magnetic field (Atkins, 1990; Wilson, 1987). Each isotope has a specific number of energy levels, determined by its spin quantum number (I). The NMR phenomenon is based on transitions and coherences between these energy states. For both $^{1}H$ and $^{13}C$, for example, the spin quantum number is I = ½, resulting in two energy levels with an energy difference designated ΔE. In a magnetic field, this energy difference makes the nuclei NMR-active. In contrast, $^{12}C$ has a spin quantum number of I = 0, corresponding to one energy level with no possibility for transitions. $^{12}C$ is therefore NMR-inactive. Atoms that are often monitored in NMR analyses include $^{1}H$, $^{13}C$, $^{15}N$ and $^{31}P$.

When the compound/sample is placed in a strong magnetic field and irradiated by a short pulse of radio waves of suitable frequency, the pulse excites all the nuclei of a specified isotope at once. At a given magnetic field strength, a particular frequency is needed to excite the nuclei of a particular isotope, therefore the NMR signals of different isotopes can be manipulated individually. In the simplest form of NMR, the nuclei can then absorb energy of the radio frequency pulse, and subsequently emit radiofrequency radiation. This radiation is collected as a function of time and the time-domain signal ("free induction decay", FID) is converted into an NMR spectrum (frequency domain) by Fourier Transformation (FT). When nuclei of a particular isotope reside in different chemical environments in the sample, several slightly different frequencies are emitted, and their difference is called "chemical shift". Thus, the NMR spectrum contains one signal for each chemical environment that is occupied by the nucleus in question. NMR is a powerful technique because the chemical shifts provide information on chemical environments, and that the signals can be quantified.

Several methods have been previously used to determine the content of unfrozen water in frozen soils, such as calorimetry, conductivity and Time Domain Reflectometry (TDR) (Spaans & Baker, 1996; Gunnink, 1989; Tice *et al.*, 1978; Anderson, 1972). NMR spectroscopy, especially $^{1}H$ NMR, has also been used in previous studies to quantify the unfrozen water in frozen matrices (Watanabe & Mizoguchi, 2002; Tice *et al.*, 1978), but several problems with the use of $^{1}H$ NMR for this purpose have been reported (Hall *et al.*, 1997). The most severe problem is the small $^{1}H$ difference in linewidth between liquid and frozen water, which is exacerbated at the high magnetic fields used in modern NMR

spectrometers, and by interference from soil components that disturb the magnetic field. However, Sparrman *et al.* (2004) recently developed a NMR method using the stable heavy hydrogen isotope $^{2}H$ (deuterium) for quantifying the content of unfrozen water in frozen soils. The $^{2}H$ NMR method enables signals from liquid water and ice to be efficiently separated, even at high fields, and is less sensitive to inherent soil disturbances.

$^{13}C$ solid state NMR is one of the most powerful techniques for characterizing SOM composition (Nelson & Baldock, 2005; Kögel-Knabner, 2000; Preston, 1996; Baldock *et al.*, 1990) since it enables SOM to be structurally characterized without requiring extraction steps that often disrupt organic matter in the soil matrix. Cross Polarization (CP) techniques are often used to detect solid state spectra because $T_1$ (relaxation times) for $^{13}C$ nucleus often are excessively long for substances in solid state NMR compared to liquid NMR (Wilson, 1987). Because the $^{1}H$ isotope is inherently more NMR-sensitive than $^{13}C$, and $^{1}H$ nuclei relax faster, higher sensitivity can be obtained in a shorter time if magnetization can be transferred from $^{1}H$ to $^{13}C$. In solid-state NMR, this is achieved by CP. CP implies that after excitation of the $^{1}H$ spins, magnetization is transferred to $^{13}C$ by a simultaneous pulse called "spin lock" on $^{1}H$ and $^{13}C$ and detected as $^{13}C$ FID. The CP effect is most pronounced in solid materials, and is attenuated by mobility of the $^{13}C$-$^{1}H$ spin system. Moreover, CP is dependent on carbon contacts with nearby protons, hence graphite (for instance) is invisible in a CP experiment. Since NMR signals of solid samples are often very broad, CP is often combined with "Magic Angle Spinning" (MAS), a technique that reduces linewidths by averaging out orientation-dependent interactions by fast sample spinning. Thus, $^{13}C$ spins in solid environments can be identified, but quantification of CP-MAS spectra requires caution since some $^{13}C$ atoms may not be effectively detected.

$^{13}C$ MAS NMR is a technique which implies direct excitation of C and the use of MAS, and allows detection of mainly soluble compounds and semisolid compounds because the fast sample rotation of MAS reduces the line widths. Complete solid compounds can be detected, but they often have broad line shapes and low intensity. This NMR method can be used for investigating microbial substrate utilization and the formation of different metabolites. In $^{13}C$ solution NMR (without MAS), more solid compounds are invisible due to their large linewidths, so only the soluble compounds are detected.

# 2 Material and Methods

## 2.1 Site descriptions

The investigated soil samples were of Spodsols or Histosols, the major soil types in the boreal region (Soil Survey Staff, 2003). The investigations were confined to the organic (O)-horizon because microbial activity mainly occurs in this horizon of boreal soils and frost in the boreal region mainly affects the surface layers. The soil samples investigated in this project were collected from six locations in the central boreal region of northern Sweden and one in the southwest of Sweden at the southern border of the boreal climate zone. The aim of the sampling strategy was to cover a wide range of the spatial distribution of boreal terrestrial soil systems, including several pine-dominated, spruce-dominated and mire sites (Figure 3). In Papers I-II, we investigated Spodosol soil samples obtained from different pine (*Pinus sylvestris, L.*) and spruce (*Picea abies, L.*) dominated sites. Each pine site was sampled at 1-3 depths (n = 9, where n is the total number of samples per forest soil type) and each spruce site at 1-2 depths (n = 5), depending on vegetation cover and O-horizon thickness. In Paper III, samples from a mixed deciduous stand (sampled at 1-2 depths; n=2) and three sub-habitats of a *Sphagnum* mire (sampled at 1-2 depths; n=6) were also included. The understory and bottom vegetation of the pine forest soils were dominated by *Calluna vulgaris* (heather), *Pleurozium schreberi* (moss), and *Vaccinium vitis idea* (lingonberry). The understory and bottom vegetation of the spruce forest soils were dominated by *Deschampsia flexuosa* (wavy hair grass), *Vaccinium vitis idea* (lingonberry), and *Vaccinium myrtillus* (blueberry). At the mire sites the understory and bottom vegetation were dominated by *Sphagnum* moss (*Sphagnum fuscum, Sphagnum balticum* and *Sphagnum lindbergi,* respectively). Detailed site and soil descriptions can be found in

the online supporting information for Paper III. In Paper IV, samples from a spruce-dominated forest site (*Picea abies, L.*) with sparse pine (*Pinus sylvestris, L.*) were collected (by bulk sampling of the upper 10 cm of the O-horizon). The understory and bottom vegetation were dominated by *Vaccinium vitis idea* (lingonberry), *Vaccinium myrtillus* (blueberry) and *Pleurozium schreberi* (moss). The soil samples from all sites were sieved (mesh size 3 mm) and coarse debris and fine roots were removed.

*Figure 3.* Photographs of one of the spruce-dominated sites (top), one of the pine-dominated sites (lower left) and Degerö Stormyr, a mire site (lower right). Photo: Mats Öquist (pine and mire sites) and Peder Blomkvist (spruce site).

## 2.2 Total water potential of unfrozen water in frozen soil (Paper I)

In Paper I, the total water potential of soil water was described as a function of a given freezing point depression in order to determine the water potential of the remaining liquid water in the frozen soil matrix. This was done by combining equations for water potential (Atkins, 1990) and freezing point depression (see Eqs. 1, 2, and 3 in Paper I). We conducted experiments at -7°C and -4°C, at which the water potentials were calculated to be -8.3 MPa ($\Psi_{TOT\ -\ 7°C}$) and -4.8 MPa ($\Psi_{TOT\ -\ 4°C}$), respectively.

### 2.2.1 Effects of particle size and osmotic potential in defined mineral soil fractions on unfrozen water in frozen soils (Paper I)

In Paper I, we first wanted to elucidate the combined effects of particle size and osmotic potential on the unfrozen water content of frozen soils. Different mineral soil fractions (ranging from coarse sand to fine silt, Table 1) were mixed with solutions of selected initial osmotic potentials (0, -1.5, -2, -3, -4.5 and -6 MPa, prepared by adding KCl to water). The fraction of unfrozen water (y) in the samples at -7°C in samples with different initial osmotic potentials (Figure 4) was described as a function of the initial osmotic potential as follows,

$$y = FUW_{0MPa} + \frac{\psi_{Oimitial}}{-8.3MPa - \psi_{M-7^{\circ}C}} \qquad \textit{(Eq. 3)}$$

where: $FUW_{0\ MPA}$ is the fraction of unfrozen water at -7°C at an initial osmotic potential of 0 MPa, i.e. with no added KCl (corresponding to the Y-axis intercept of the function); $\Psi_{Oinitial}$ is the initial osmotic potential (MPa) prior to freezing, i.e. the added amount of KCl; and $\Psi_{M\text{-}7°C}$ is the contribution of the matric potential (MPa), i.e. the effect of particle size on the unfrozen water at -7°C. The slope of Eq. (3) is equal to $1/(-8.3MPa-\Psi_{M\ -\ 7°C})$ and the influence of particle size (matric potential) on unfrozen water content in the samples should be manifested as differences in the slope of the regression. The unfrozen water content in the frozen samples was measured by $^{2}H$ NMR spectroscopy (Sparrman *et al.*, 2004).

### 2.2.2 Contributions of matric and osmotic potentials to the unfrozen water contents of frozen boreal pine and spruce forest soil samples (Paper I)

In Paper I we also wanted to determine the relative contributions of matric and osmotic potentials to the unfrozen water content in frozen samples of the O-horizon of boreal forest soils. To determine water-holding capacities (matric potentials) of the samples, soil water retention curves were determined, including data acquired when pressures of -0.5, -1.5, -3 and -6 MPa were applied, for each soil (Paper I). A steel pressure chamber, with a ceramic plate, was used to determine the soils' water-holding capacity at -0.5 and -1.5 MPa and vapour equilibrium chambers (Cambell & Gee, 1998) were used to determine their water-holding capacity at -3 and -6 MPa. See Paper I, for details regarding the soil water measurements.

As mentioned earlier, liquid water must have a total potential of ~ - 4.8 MPa, to be in equilibrium with pure bulk ice at -4°C. The contribution of the matric potential to the water potential at -4°C in the forest soils was determined by combining $^{2}$H NMR-measured values of the amount of unfrozen water and exponential functions derived from the soil water retention curves (Paper I). The contribution of osmotic potential ($\Psi_O$) to the amount of unfrozen water at -4°C in each case was then calculated as the difference between the estimated matric potential and the total water potential at -4°C (see Paper I).

### 2.2.3 Equivalent pore sizes with unfrozen water in relation to the size of soil microorganisms (Paper I)

We wanted to estimate the sizes of the pores in which unfrozen water exist under frozen conditions in order to elucidate if pores could be large enough to harbor active microbial cells. These pore sizes depends on the pore size distribution and water holding capacity of the soil. The definition of pore size equivalents is that for every natural pore, you can find a circular pore that is drained at the same tension. Thus, the volume of water removed from a given volume of soil at a specified tension represents the volume of pores indicated by that tension (Vomocil, 1965). Using the law of capillarity (Hillel, 2005; Baver *et al.*, 1972), together with the determined matric potentials at -4°C, we estimated the maximum diameter of such pores for each of the samples at -4°C (see Eq. 6 in Paper I). This relationship assumes that the pores are circular in cross-section, but can be used to theoretically explain differences among samples with respect to pore size distribution, unfrozen water content, or thickness of the water film, even if their pores are not circular (Andersson & Wiklert, 1972). These

pore size equivalents were also evaluated in Paper II to investigate correlations with SOM composition.

## 2.3 Influence of soil freezing on the temperature response of soil $CO_2$ production (Paper III)

In Paper III we investigated the importance of unfrozen water for the temperature response in heterotrophic $CO_2$ production. The response in heterotrophic $CO_2$ production to different incubation conditions can be described the by the ratio of two $CO_2$ production rates ($Q$) at different temperatures. The temperature response of soil $CO_2$ production changes across the freezing point and is affected not only by temperature, but also (as we hypothesized) by the unfrozen water content through its influence on microbial activity in the frozen matrix. Thus, any model describing the observed response should include an Arrhenius-based function (expressing the direct temperature response) and a function expressing the constraint imposed by the reduced water content. In Paper III we proposed a model factoring out the contribution of unfrozen water to the estimated temperature response so a true biochemical temperature response for frozen soils could be determined. The ratio $Q$ ($k_{+4°C}/k_{-4°C}$) was expressed as follows,

$$Q = Q_T \cdot e^{\frac{Xs}{WC_T - WC_{\lim}}}$$

*(Eq. 4)*

where $Q_T$ is the Arrhenius-based biochemical temperature response representing the production response asymptote at non-limiting water content, $WC_T$ is the unfrozen water content at -4°C, $WC_{lim}$ represents the water content at which $CO_2$ production ceases and the response approaches infinity, and Xs describes the shape of the curve connecting the two asymptotes.

## 2.4 Soil incubations (Paper III and IV)

In Papers III and IV, soil incubation experiments were performed to measure the soil $CO_2$ production at different temperatures (specified below). In these experiments, soil was transferred to gas-tight glass bottles, the bottles were then incubated at chosen temperatures for a chosen time, and $CO_2$ was withdrawn from the glass bottles at regular intervals to

determine production rates. In Paper III, incubation experiments were carried out at +4°C and -4°C to determine the $CO_2$ production rates at these temperatures in order to obtain a response variable (Q; see section 2.3 and Paper III) across the freezing point of the bulk soil water. In Paper IV, a solution of $^{13}$C-labelled glucose, $(NH_4)_2SO_4$ and $K_2HPO_4$ (C, N, P, 1:1/13:1/18) was added to the samples before the incubation experiments started. The added amount of $^{13}$C glucose corresponded to 40 mg per g SOM and the water content to 550% $H_2O$ per g SOM. The amounts of $^{13}$C glucose solution added to the bottles and final soil water contents were chosen to correspond to optimal conditions for soil microbial activity in similar soil samples (Schnürer, 2006; Ilstedt et al., 2000). The preparations were incubated at +4°C, +9°C, -4°C and -9°C to determine the microbial utilization of $^{13}$C glucose and hence the rates of $^{13}CO_2$ production and synthesis of various $^{13}$C-labelled compounds in the soil (Paper IV). Control samples to determine non-biological $CO_2$ production rates at the investigated temperatures were obtained by autoclaving samples in Paper III and by sterilizing soil samples with 0.5% $NaN_3$, corresponding to ~ 7.7 mmol $NaN_3$ per kg soil (Wolf et al., 1989), in Paper IV. See Papers III and IV for details about the incubation experiments.

## 2.5 NMR spectroscopy

### 2.5.1 $^2$H NMR measurements of unfrozen water (Papers I and III)

In Paper I, and III $^2$H NMR was used to determine the contents of unfrozen water in frozen soils at -4°C and -7°C. In one of the spruce soils (soil K), the fractions of unfrozen water were determined in a temperature range down to -90°C. The soils were packed in 10 mm NMR tubes and deuterated water ($^2H_2O$) was added to the samples, to a final concentration of ~ 2% $^2H_2O$. NMR spectra of the samples were then acquired using a Bruker Avance DRX 500 spectrometer. The unfrozen water contents of the frozen soil samples were analyzed using several versions of the $^2$H NMR method by Sparrman *et al* (2004). See Papers I and III for details about the $^2$H NMR measurements of unfrozen water.

### 2.5.2 Solid state CP-MAS NMR characterization of SOM composition (Paper II)

In Paper II, we used CP-MAS NMR to characterize the SOM composition of boreal forest soils. $^{13}C$ NMR spectra of SOM usually contain signals in the range 0-190 ppm (Smernik, 2005), and the following chemical shifts were used to identify the chemical composition of the SOM of the soils (Nelson & Baldock, 2005; Smernik, 2005; Hannam *et al.*, 2004; Kögel-Knabner, 2000; Wilson, 1987): 0-45 ppm (alkyl C), 45-60 ppm (methoxy/N-alkyl C), 60-110 ppm (O-alkyl C), 110-145 ppm (aromatic C), 145-160 ppm (O-aromatic C) and 160-190 ppm (carbonyl C). All spectra presented in Paper II were obtained using a Varian/Chemagnetics CMX400 spectrometer operating at a $^{13}C$ spectral frequency of 100.72 MHz. See Paper II for details about the CP-MAS NMR settings.

### 2.5.3 $^{13}C$ MAS NMR and $^{13}C$ solution NMR determination of microbial glucose-C utilization and synthesis of C compounds in soil (Paper IV)

In Paper IV, we used $^{13}C$ MAS NMR and $^{13}C$ solution NMR spectroscopy to monitor the microbial $^{13}C$ glucose turnover in soil at +9°C, +4°C, -4°C and -9°C. All MAS NMR spectra were acquired with a Bruker Avance DRX 500 spectrometer and samples were spun at 4 kHz in a MAS probe. Control samples to determine non-biological activity at the investigated temperatures were also analyzed by NMR (as described in 2.4). In addition to MAS NMR, one sample incubated at -4°C was extracted with $D_2O$ after disrupting the cells, and analyzed by $^{13}C$ solution NMR to identify the $^{13}C$ MAS peak at 63.6 ppm (glycerol). See Paper IV for details about the $^{13}C$ MAS and solution NMR settings.

## 2.6 $CO_2$ measurements

In Papers III and IV, the concentrations of total $CO_2$ and $^{13}CO_2$ in the incubation bottles were determined by analyzing gas samples withdrawn from their headspaces. Total $CO_2$ concentrations ($^{12}CO_2$+$^{13}CO_2$) were measured by a GC-FID system (Perkin Elmer; Waltham, Massachusetts, USA), equipped with a methanizer operating at 375°C, using He as carrier gas. This is a chromatographic technique in which a gas sample is injected onto the separation column in a carrier gas flow. The $CO_2$ is then separated from other compounds due to differences in the strength of its interactions with the column's stationary phase. The $CO_2$ is then converted in the methanizer to $CH_4$, which is detected by a FID (Flame Ionization

Detector). In Paper IV, the fraction of $^{13}C$ in $CO_2$ gas formed by microbial activity was determined using an isotope ratio mass spectrometer (GC-IRMS; Model 2020 Analyzer, Europa Scientific Ltd., Crewe, Cheshire, UK) interfaced to a sample preparation unit (Cryoprep, SerCon Ltd, Crewe, Cheshire, UK). See Paper III for details about the GC-FID settings and Paper IV for details about the $^{13}CO_2$ measurements.

## 2.7 Statistics and data evaluation

In all of the Papers, differences between samples and treatments were deemed to be significant if $p < 0.05$. The $CO_2$ production rates at -4°C (presented in Paper III) were also used in Papers I and II. Linear regression, principal component analysis (PCA), and projections to latent structures by means of partial least squares (PLS) were all used for data evaluation. $^{13}CO_2$ production rates reported in Paper IV were obtained by linear regressions. Statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, IL, USA), Simca-P, version 10.5 (Umetrics, Umeå, Sweden), Minitab 15 and Excel (Microsoft Office Excel 2003).

In Paper II we used PLS to evaluate how the chemical composition of SOM (X data, measured by CP-MAS NMR) affected the amount of unfrozen water, pore size equivalents and heterotrophic microbial activity in frozen boreal forest soils (Y data, from Paper I and III). PLS is a regression extension of PCA that is used to relate two data matrices, X and Y, to each other by a linear multivariate model (Eriksson *et al.*, 2001). The performance of a PLS model is described by the amount of variance explained ($R^2$) and its predictive power ($Q^2$). The major advantage of PLS, compared to PCA, is that each row of a data table corresponds to two points rather than a single point, i.e. one in X-space and one in Y-space. The number of components in the PLS models was added as long as both $R^2$ and $Q^2$ increased.

In Paper IV, changes in the allocation of C to catabolic and anabolic processes over time were evaluated using data on residual $^{13}C$ glucose, synthesized $^{13}C$ compounds (measured by $^{13}C$ MAS NMR), and $^{13}CO_2$ concentrations at each of the investigated temperatures. The added amount of glucose was used as a reference (100%). The proportions of the following synthesized $^{13}C$ compounds -- glycerol (only including C1 and C3), the acyl chain of phospholipids, polymeric carbohydrates (as glycogen), protein compounds and ethanol -- were determined at the times when 50% of the glucose had been consumed (50% glucose). This point in time was estimated by linear interpolation between the two closest measurement

points between which half of the glucose remained. Carbon use efficiency (CUE; the total amount of synthesized $^{13}C$ compounds/amount of $^{13}CO_2$) and "growth" index (defined as the amount of phospholipids/amount of $^{13}CO_2$) were determined at +9ºC, +4ºC, and -4ºC, at the time when 50% glucose was left. In Paper IV, the degree of unsaturation and average length of the NMR-determined phospholipids were also determined at +9ºC, +4ºC and -4ºC (see Paper IV for calculations).

# 3 Results and Discussion

## 3.1 Soil physical factors controlling the content and distribution of unfrozen water and related C mineralization of frozen soils

### 3.1.1 Influence of matric and osmotic potentials on the unfrozen water content of frozen soils

In Paper I, we investigated the effects of different mineral soil fractions (with varying particle sizes, Table 1) and different osmotic potentials on the fractions of unfrozen water at -7°C (Figure 4). At this temperature no unfrozen water was detected in the mineral soil fractions with an initial soil water potential of 0 MPa. Thus, the influence of matric potential (particle size) appeared to be negligible for these mineral soil fractions. However, the ability of the mineral soil fractions to contain unfrozen water at -7°C increased as their initial osmotic potential became more negative (Figure 4). The theoretical fraction of unfrozen water in an ideal solution, in which all of the solutes are assumed to be concentrated upon freezing, is illustrated by the black line in Figure 4. The slopes of the linear functions obtained by regression (Table 1) did not significantly vary among the investigated fractions (Dixons Q-test; (Rorabacher, 1991) and the average regression coefficient of the equations (-0.118) did not significantly differ (95% t-test) from the regression coefficient of the ideal line (-0.121). These findings show that the investigated particle sizes had negligible effects on the fraction of unfrozen water at -7°C in this model soil system, even when the osmotic potential declined.

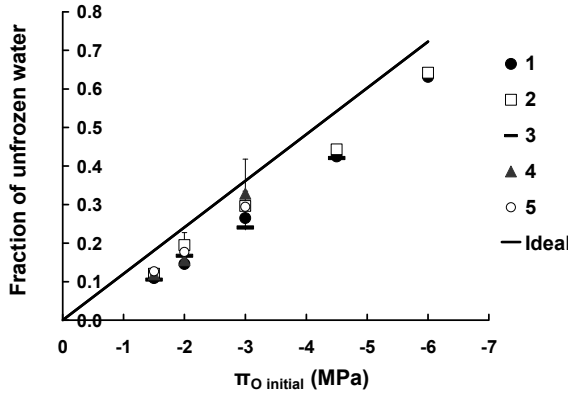



*Figure 4.* The fraction of unfrozen water, measured by NMR, as a function of the initial osmotic potential $\Psi_{O\ initial}$ (added KCl) of the soil solution prior to freezing (-7°C) for each of the five mineral soil fractions (1-5). Error bars indicate SE. Some replicates did not freeze at -7°C due to hysteresis and are therefore excluded. The theoretical fraction of unfrozen water in an ideal solution, in which all of the solutes are assumed to be concentrated upon freezing, is illustrated by the black line.

However, effects of particle size on unfrozen water contents of soils have been found in other studies (Romanovsky & Osterkamp, 2000; Spaans & Baker, 1996; Tice *et al.*, 1978). The effects in these studies may be related not only to variations in particle size distribution, but also to solutes in the soil water affecting the osmotic potential. Our samples contained no clay, hence the observed variation in unfrozen water in the mentioned studies (Romanovsky & Osterkamp, 2000; Spaans & Baker, 1996; Tice *et al.*, 1978) may also have emanated from water-clay interactions.

Table 1
Soil type, particle size and relationship between initial osmotic potential and the fraction of unfrozen water in the investigated mineral soil fractions.

| I.D | Type | Particle size (mm) | Regression eq. [a] | $r^2$ | p |
|---|---|---|---|---|---|
| 1 | Coarse sand | 0.5-1.0 | y = -0.116x - 0.079 | 1.00 (n=5) | < 0.001 |
| 2 | Sand | 0.2-0.6 | y = -0.112x - 0.042 | 1.00 (n=5) | < 0.001 |
| 3 | Silt | 0.02-0.06 | y = -0.103x - 0.049 | 0.99 (n=4) | 0.005 |
| 4 | Medium silt | 0.006-0.06 | y = -0.144x - 0.111 | 0.97 (n=3) | 0.1 |
| 5 | Fine silt | 0.002-0.006 | y = -0.112x - 0.044 | 1.00 (n=3) | 0.02 |

a: The fraction of unfrozen water (y) as a function of the initial osmotic potential x (MPa).

Since water-holding properties are important for the ability of a soil to retain unfrozen water, I also determined the relative contributions of matric and osmotic potentials to the unfrozen water in frozen (-4°C) boreal pine and spruce forest soils (Paper I). The SOM contents of samples from the pine- and spruce-dominated sites ranged from 55 to 97 %. The contributions of matric potential to the total water potential (-4.8 MPa at -4°C) of the forest soils ranged from – 1.5 to -3.9 MPa, and the contributions of osmotic potential from -0.9 to -3.3 MPa, corresponding to ca. 20-70 % of the total water potential (Paper I). Estimated unfrozen water contents retained at a matric potential of -4.8 MPa (i.e. at -4°C) were also determined in Paper I, from the exponential function of the water retention curves. These estimated values were consistently lower than those measured in the frozen soil samples at -4 °C (Paper I), suggesting that this difference in water content represented the amount of unfrozen water emanating from the osmotic potential, corresponding to around 17-46 % of the measured unfrozen water contents.

It has been suggested that the contribution of osmotic potential to the total water potential is negligible in frozen soils (Suzuki, 2004). However, the soils studied by Suzuki et al (2004) contained significant amounts of clay, and hence could conceivably have had very high matrix water-holding capacities. Nonetheless, in the organic horizon of boreal forest soils, our results show that both matric and osmotic potentials contribute to the unfrozen water content. However, in unfrozen soil, osmotic soil water potentials are low (~-17 to -113 kPa) compared to the total water potentials of frozen soils, meaning that solutes in the liquid water must be concentrated as a result of freezing. This supports the concept that reductions in water contents induced by freezing reduce the thickness of the water film, thereby increasing osmotic concentrations in the remaining liquid water (Torrance & Schellekens, 2006).

In summary, I concluded that the solutes in natural soil water must be concentrated to generate sufficient osmotic potentials to yield unfrozen water in frozen soils. The generally similar contributions of matric and osmotic properties to the soil water potential in frozen soils is conceptually important for attempts to relate unfrozen water content to processes such as C mineralization because a relatively high contribution of the latter would increase the thickness of unfrozen soil water films and pores with liquid water that could harbour active microbial cells.

### 3.1.2 Effect of pore sizes on unfrozen water contents and biogenic $CO_2$ production of frozen soils

The sizes of the pores where the unfrozen water existed in the investigated frozen boreal soil samples ranged from 0.08-0.14 μm and were found to strongly correlate to the biogenic $CO_2$ production at -4°C ($p < 0.001$, $r^2 = 0.71$, Paper I). When SOM degrades, the bulk density, proportions of smaller pores and water holding capacity all increase (Päivänen, 1973), which also affect the capacity of the soil to retain unfrozen water. Not only the amount of liquid water but also the pore size equivalents in which the unfrozen water resided correlated with the biogenic $CO_2$ production at -4°C. This can be explained by that the pore size distribution also is a determinant for which microorganisms that can occupy the pores. Thus, the results show that the determined pore sizes in the frozen soils at -4°C were sufficiently large to permit occupation by viable soil microorganisms, the smallest of which are suggested to have ca. 0.05 μm diameters (Panikov *et al.*, 2005).

The lowest temperature at which unfrozen water could be detected in a boreal spruce soil sample was around -80°C (Figure 5), supporting previous observations of microbial activity at extremely low temperatures (Panikov & Sizova, 2007; Panikov *et al.*, 2006), as long as the microorganisms can tolerate the low soil water potentials required to keep water in its liquid state. There are reports in the literature that some soil microorganisms can tolerate water potentials down to -65 MPa (Harris, 1981), corresponding to a temperature of around -54°C. The contribution of matric potentials to the unfrozen water content at -20°C and -40°C were determined using the measured contents of unfrozen water and extrapolations of the water retention curves in Paper I. This resulted in matric potentials of around -4.7 MPa and -7.2 MPa, respectively, corresponding to pore size equivalents of ca. 0.063 μm (-20°C) and 0.041 μm (-40°C). The relatively modest change in matric potential as water contents decrease also show that the relative contribution of the osmotic potential increases as temperatures drops. Although it may not be thermodynamically rigorous to discuss freezing point depressions > 30°C as being solely due to the pore size distribution, these estimates still provide an indication of the pore size required to retain liquid water. Hence, some microorganisms (Panikov *et al.*, 2005) may occupy pore spaces of frozen soil even down to around -40°C, and this further supports the occurrence of microbial activity at extremely low temperatures (Panikov & Sizova, 2007; Panikov *et al.*, 2006; Rivkina *et al.*, 2004). The potential interactions between the influential factors also complicate attempts to determine a minimum threshold for

metabolism (Price & Sowers, 2004) based solely on temperature. In conclusion, the larger the pores in which liquid water resides, the greater the microbial activity and C mineralization that can be sustained in frozen soils.

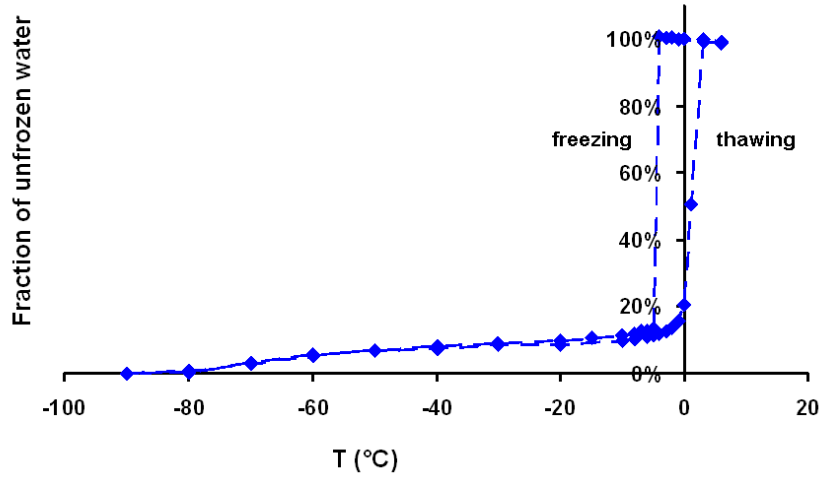



*Figure 5.* Soil freezing curve illustrating the fraction of unfrozen water as a function of temperature for a spruce soil sample (soil K, see Papers I, II and III). Unfrozen water was detected down to around -80°C in the O-horizon of this boreal spruce forest soil.

### 3.1.3 Effect of SOM composition on unfrozen water, pore size equivalents and microbial $CO_2$ production

Since SOM influences the water-holding properties of soils (Andersson & Wiklert, 1972) we hypothesized that it also would influence the ability of the investigated forest soils to retain unfrozen water. The chemical shift regions used to characterize the SOM by CP-MAS NMR are illustrated in Figure 6. The chemical composition of the SOM in the analyzed soil samples corresponded well to the composition reported in previous studies of forest soils (Preston *et al.*, 2000; Preston, 1996). Interestingly, pine and spruce forest soils could not be evaluated together (data not shown) and had to be evaluated separately. However, for both systems the composition of SOM was found to be significantly related to unfrozen water content, pore size equivalents with unfrozen water, and the heterotrophic microbial activity in the frozen soil samples.

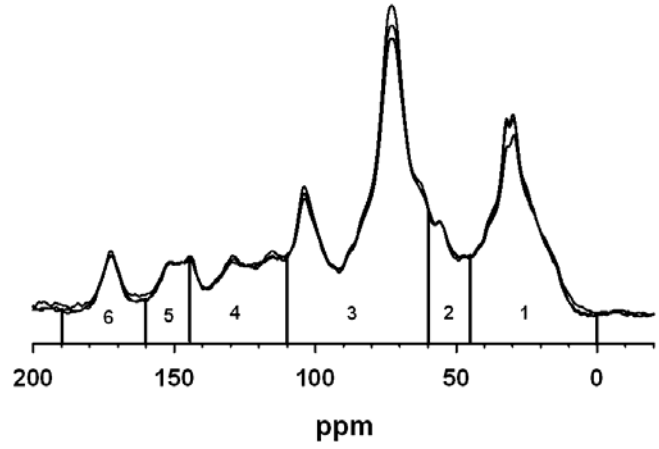



*Figure 6.* Representative CP-MAS NMR spectra of replicate samples of a pine-dominated forest soil (n=3), with indications of the following chemical shift regions: 1 (0-45 ppm), alkyl C; 2 (45-60 ppm), methoxy/N-alkyl C; 3 (60-110 ppm), O-alkyl C; 4 (110-145 ppm), aromatic C; 5 (145-160 ppm), O-aromatic C; and 6 (160-190 ppm), carbonyl C.

A strong positive correlation between aromatic C compounds and unfrozen water was observed in the data obtained from all the pine soil samples, representing a wide variety of sites (Figure 7, and Paper II). When SOM is degraded a shift in the O-alkyl C content occurs and the O-alkyl C intensity decreases (Kögel-Knabner, 2000; Preston *et al.*, 2000), while the relative intensity of all other regions increases, in particular the alkyl C region. These changes can be explained by the preferential degradation of carbohydrates and the accumulation of more recalcitrant material (Kögel-Knabner, 2000). The findings are also consistent with an observed increase in the fraction of alkyl C relative to O-alkyl C in the humifaction gradient, derived from sampling at different depths. However, the correlation between aromatic C compounds and unfrozen water indicated that the quality and input of litter, in addition to its degree of humification, may also influence the unfrozen water content.

Analyses of samples collected from different depths did not show a large increase in aromatic C compounds with depth in the pine soils, but a shift from O-alkyl to alkyl C was detected, suggesting that the proportion of recalcitrant material increases with depth, in accordance with previous studies, e.g. Preston *et al* (2000). Further, the increase of recalcitrant material coincided with increases in soil density and effects on pore size distribution that result from SOM degradation and enhance soil water-holding capacities (Päivänen, 1973). Thus, aromatic C compounds of SOM

can retain more unfrozen water at a given temperature than O-alkyl C compounds of SOM (e.g. carbohydrates). This can be explained by the effect of more recalcitrant compounds on pore size distribution, as described for instance by Päivaänen (1973) and effects induced by the soil matrix, e.g. adsorption and surface properties.

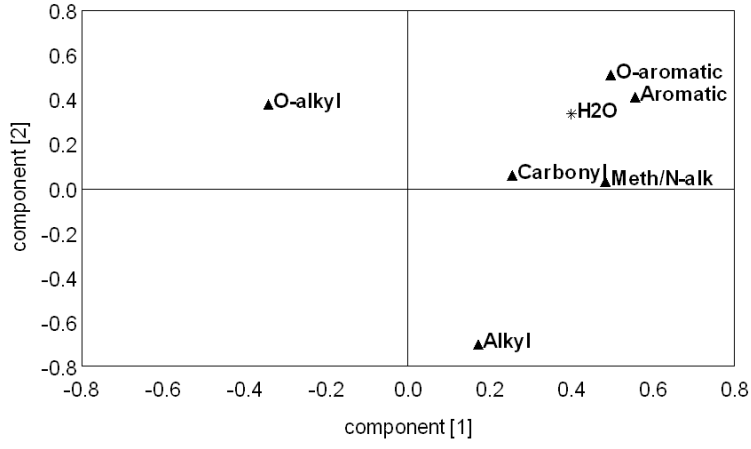
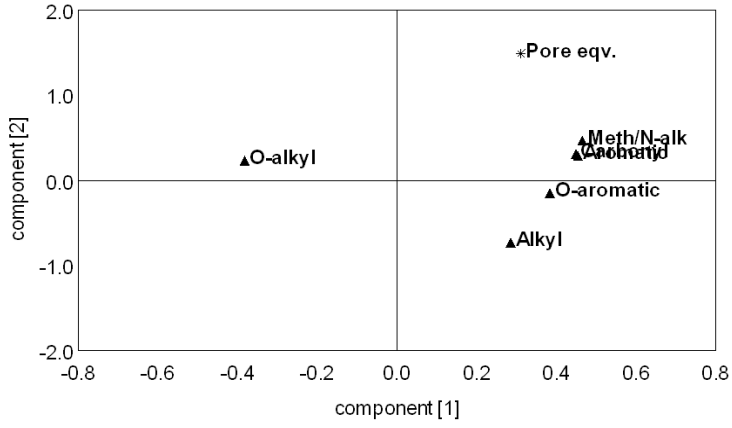
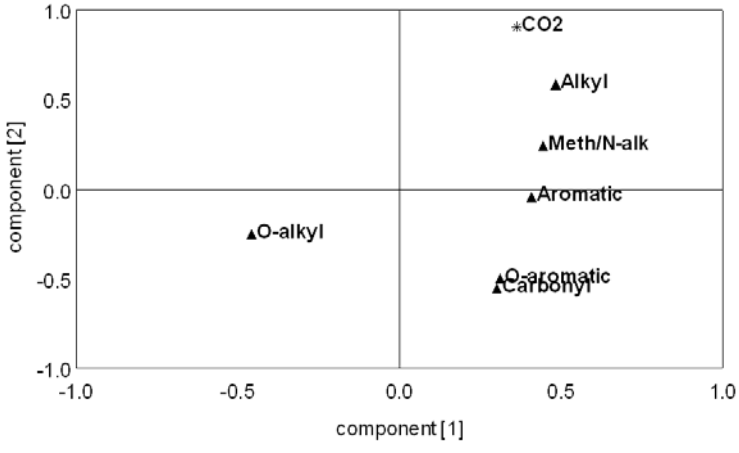



*Figure* 7. PLS loading plot for SOM components (independent X-variables) and unfrozen water content at -4°C (dependent Y-variable) of the pine forest soils (n=9). $R^2$ (Y) = 0.71 (component 1 $R^2$ = 0.58, component 2 $R^2$=0.13), $Q^2$ = 0.56. "Meth/N-alk" is methoxy/N-alkyl C and "H2O" is g unfrozen water content at -4°C per g SOM.

PLS analysis revealed that unfrozen water contents of the frozen pine forest soil samples were negatively correlated with the content of O-alkyl C compounds, i.e. carbohydrates (Figure 7). It is worth mentioning that the osmotic potential, i.e. concentration of solutes (which may be soluble carbohydrates), affect the unfrozen water contents of frozen soils. However, the amounts of O-alkyl C compounds that are soluble in water as soil freezes are probably low compared to the total amount of O-alkyl C compounds, which includes more polymeric carbohydrates, e.g. cellulose. Therefore, the total O-alkyl C content (mainly carbohydrates) in the organic horizon of pine soils appears to have relatively weak effects on the capacity of soil to retain unfrozen water compared to the content of compounds rich in aromatic structures.




a)




b)

*Figure 8a and b.* PLS loading plots for SOM components (independent X-variables) and equivalent pore diameter at -4°C (dependent Y-variable, Fig. 8a) and microbial $CO_2$ production at -4°C (dependent Y-variable, Fig 8b) of the spruce forest soils. a) $R^2$ (Y) = 0.89 (component 1 $R^2$ = 0.53, component 2 $R^2$=0.36), $Q^2$ = 0.63. b) $R^2$ (Y) = 1.0 (component 1 $R^2$ = 0.71, component 2 $R^2$=0.29), $Q^2$ = 0.97. "Meth/N-alk" is methoxy/N-alkyl C, "Pore eqv." is pore size equivalents, and "CO2" is the microbial $CO_2$ production per g SOM and day (at -4°C).

In the spruce forest soils, the unfrozen water contents did not appear to be correlated to the organic chemical characteristics of SOM. However, the determined pore sizes in which unfrozen water resides at -4°C were positively correlated to the methoxy/N-alkyl C content (PLS analyses, Figure 8a). As for the pine soils, this relationship indicated that differences in litter input and degree of humification affect the pore size distribution in

these soils, since methoxy C in SOM can be largely attributed to lignin (Lorenz *et al.*, 2000).

The biogenic $CO_2$ production rates at -4°C of the spruce soils correlated negatively with the abundance of carbohydrates, i.e. O-alkyl C (PLS, Figure 8b, and Paper II), in strong contrast to the general promotion of microbial activities by carbohydrates in SOM under unfrozen conditions (Bosatta & Ågren, 1999). Hence, carbohydrates are often considered to have higher substrate quality (i.e. to be easier to degrade) than (for instance) lipids or aromatics, which are considered as low quality organic matter (Bosatta & Ågren, 1999). However, the NMR-measured O-alkyl C content (carbohydrate constituents) is most probably represented by a combination of both easily degradable and more recalcitrant carbohydrates. As previously described, O-alkyl C can be considered more prone to decomposition compared to alkyl C, aromatics and phenolic C. Therefore, the results demonstrate that the organic matter composition of the soils affects the heterotrophic microbial activity in several interacting ways; not only as a carbon and energy source, but also as a physical determinant of water availability under frozen conditions.

The biogenic $CO_2$ production rates in the frozen spruce forest soil samples (-4°C) were also positively related to both the methoxy/N-alkyl C and aromatic C contents in the SOM (PLS, Figure 8b, and Paper II). This may also be counter-intuitive, since carbohydrates are considered more prone to mineralization than more recalcitrant compounds. However, this can also be explained by the influence of recalcitrant SOM fractions on soil-water interactions, the pore size distribution and (hence) the unfrozen water content and $CO_2$ production. The strong correlation between biogenic $CO_2$ production rates at -4°C in frozen forest soils and sizes of pores where unfrozen water resides (Paper I) is likely to emanate from factors such as the aromatics' effect on the pore size distribution.

However, the SOM parameter that $CO_2$ production rates at -4°C in the spruce soil samples were most strongly (positively) correlated to was the content of alkyl C (Figure 8b, and Paper II). Alkyl C largely originates from recalcitrant plant biopolymers, such as cutin and surface waxes, but some originates from newly produced microbial biomass (Nelson & Baldock, 2005; Lorenz *et al.*, 2000; Preston, 1996; Baldock *et al.*, 1990). Soil samples with high levels of alkyl C, mainly consisting of amorphous polymethylene, have been found to have high sorption coefficients (Simpson & Johnson, 2006), implying that alkyl C contents affect properties such as mobility (of the SOM) and sorption (having high affinity for hydrophobic organic compounds). This would have consequences for the

pore size distribution, surface properties and (thus) interactions between soil and water. Increasing proportions of alkyl C with decreasing particle size have also been found in analyses of particle size fractions from cultivated grassland soils (Preston *et al.*, 1994b), with consequent effects on soil density and water retention. Although we found no direct correlation between unfrozen water and alkyl C, the relative content of alkyl C was correlated to the microbial $CO_2$ production rates at -4°C (Figure 8 and Paper II). Previous findings that the proportions of alkyl C increase as SOM degrades (e.g. Kögel-Knabner, 2000) and soil density increases as decomposition proceeds (Päivänen, 1973), further suggest that alkyl C affects the pore size distribution and unfrozen water contents. This could also explain the observed correlation between soil microbial $CO_2$ production rates at -4°C and pore size equivalents in which unfrozen water resides (Paper I).

To summarize, the observed differences between pine and spruce soils suggest that vegetation cover and input of litter influenced the composition of SOM in the samples. In the pine soils, the relationship between aromatic compounds and unfrozen water was most important, while both aromatics and alkyl C compounds strongly influenced pore size equivalents with unfrozen water and $CO_2$ production rates of frozen spruce soils. I concluded that the SOM characteristics quantified by solid state NMR mainly appear to be strongly related to soil physical parameters, such as pore size distributions and unfrozen water contents. The latter then affect microbial activity via their effects on processes such as substrate diffusion. Thus, in soils with high organic matter contents the SOM composition is a major determinant of pore size distribution and the soils' ability to retain unfrozen water at sub-zero temperatures. This has direct effects on winter microbial $CO_2$ production and C mineralization in frozen soils.

## 3.2 $CO_2$ temperature response of frozen soils and its relationship to unfrozen water content

The studies described above showed that contents of unfrozen water in frozen soils are related to water potential, equivalent pore sizes filled with unfrozen water, and composition of SOM (Papers I and II). In addition, some of the main soil physical factors regulating the content of unfrozen water and, indirectly, the C mineralization of frozen soils have been identified and discussed (section 3.1). The next step was to investigate more direct couplings between unfrozen water and microbial processes, and the role of unfrozen water on observed temperature responses of frozen soils was investigated in Paper III.

The determined $CO_2$ production rates in Paper III ranged from 215 to 1520 μg $CO_2$ (g SOM day)$^{-1}$ at +4°C and from 15 to 300 μg $CO_2$ (g SOM day)$^{-1}$ at -4°C. Hence, the Q value of the $CO_2$ production response to changes between these temperatures ranged from 3.4 to 16.2, and the observed temperature response per 10°C (observed $Q_{10} = Q^{10/8}$) ranged from 4.6 to 36.4. Those values are higher than values obtained from measurements of unfrozen systems, but are consistent with previous observations of temperature responses in frozen samples (Panikov *et al.*, 2006; Elberling & Brandt, 2003; Mikan *et al.*, 2002). However, plotting the response of soil $CO_2$ production between +4°C and -4°C as a function unfrozen water content at -4°C (Eq. 4), revealed a strong dependence on water availability (Figure 9).

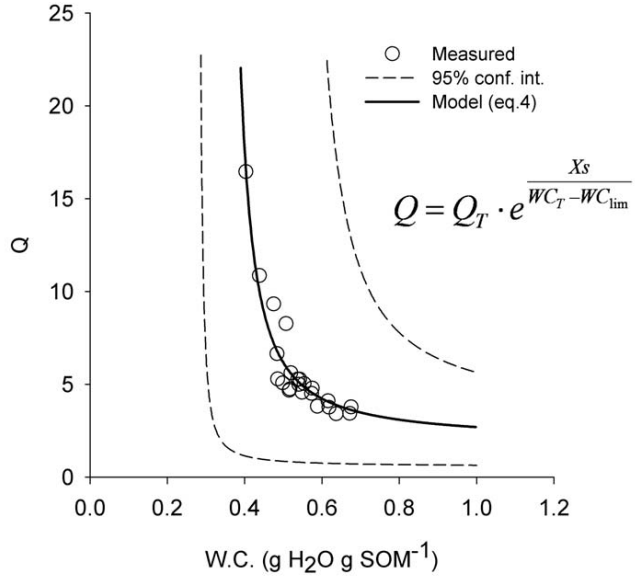



*Figure 9.* The $CO_2$ temperature response between +4 and -4°C (Q) as a function of the unfrozen water content (W.C.) in frozen soils.

An asymptotic relationship between the $CO_2$ production response and available liquid water was observed. A hyperbolic function expressing Q as a product of an Arrhenius-based function, and a function containing the constraints induced by liquid water, was therefore fitted to the data ($R^2$ = 0.92, Eq. 4; Figure 9). The X-asymptote, $WC_{lim}$, represents the water content at which the microbial activity is terminated due to water

limitations and the response (Q) reaches infinity. The Y-asymptote, $Q_T$, represents the true biological temperature response. By factoring out the effect of water availability at -4°C, using this response model, biochemical $Q_{10}$-values in the range of 1.4 - 2.6 were obtained for these samples

The obtained biochemical $Q_{10}$-values demonstrated that variations in temperature responses of $CO_2$ production and high $Q_{10}$-values observed in frozen soils (Monson *et al.*, 2006; Elberling & Brandt, 2003; Mikan *et al.*, 2002) are a result of small differences in unfrozen water contents, which depend on the soil's capacity to retain unfrozen water. Other factors proposed in the literature, such as shifts in species composition or substrate use (Schimel & Mikan, 2005; Schadt *et al.*, 2003), may have some influence on temperature responses of frozen soils, but these factors are dependent on the presence of unfrozen water. The estimated $Q_{10}$-values for the biochemical temperature response ($Q_T$; 1.4 - 2.6) in the samples agree well with true biogeochemical temperature responses (Davidson & Janssens, 2006; Davidson *et al.*, 2006). Thus, I concluded that the soils ability to retain unfrozen water, and the actual unfrozen water content at temperatures below 0°C, are major determinants for the change in $CO_2$ production with changing temperatures in frozen soils.

## 3.3 Microbial activity in frozen soils – can microorganisms both grow and respire under frozen conditions?

As mentioned above, some studies (e.g. Schimel *et al.*, 2007) have discussed the possibility if microorganisms may grow at sub-zero temperatures, while other studies have reported high amounts of soil biomass during winter (Schadt *et al.*, 2003). Therefore, it is important to determine, definitively, whether the microbial population only engages in catabolic metabolism at sub-zero temperatures, or if growth (anabolism) is possible under frozen conditions. To address this issue we investigated the microbial utilization of $^{13}C$-labelled glucose in a boreal spruce forest soil at temperatures ranging from +9°C to -9°C to determine whether it was used solely for catabolic processes or for both catabolic and anabolic processes (Paper IV).

### 3.3.1 Microbial utilization of glucose at low temperatures for $CO_2$ production and growth

$^{13}CO_2$ production in soil samples was measured during incubations at various temperatures to monitor the use of $^{13}C$ glucose for catabolic processes (Paper IV). The production of $CO_2$ was found to display a two-phase behaviour (see phases I and II, Figure 10), and two rates of $^{13}CO_2$

were extracted for each of the incubation temperatures. Accordingly, it is well known that microbial $CO_2$ production can be divided into different phases after addition of both a C-source and limiting nutrients (Ilstedt *et al.*, 2003; Nordgren *et al.*, 1988). See Paper IV for $^{13}CO_2$ production rates.

At all investigated temperatures a lag-phase (phase 1) was observed before the $CO_2$ production increased (phase 2; see Figure 10 and Paper IV). At above-zero temperatures phase 1 lasted for about two days before the rate of $CO_2$ production increased. At -4°C phase 1 lasted about 60 days and at -9°C about 100 days. The soil $CO_2$ production rates in phase 2 at -4°C and -9°C were 11 and three times higher, respectively, than the initial rates in phase 1. In the unfrozen soils, the highest $^{13}CO_2$ rates were ca. four (+9°C) and three (+4°C) times higher than the initial rates.

Winter soil respiration has been shown to respond rapidly to increased labile C availability (Brooks et al., 2005), which is consistent with our results, since utilization of the added glucose commenced within days at -4°C. However, no strong response in $CO_2$ production was evident until after 60 days. The fact that the microbial community was able to adapt to a colder environment (manifested by the shift between phases 1 and 2) and responded to increased labile C availability, indicates that more short-term incubation experiments may be inadequate for investigating the potential for low temperature biogeochemistry and its environmental controls. The $^{13}CO_2$ production rates at -9°C were generally lower than those at -4°C (Paper IV). However, at -9°C, an increase in the $^{13}CO_2$ production rate was detected after about 80-100 days, and a similar trend to that seen at -4°C was observed (Paper IV). These findings indicate that $CO_2$ production rates at -9°C could have been higher if the samples had been incubated for a longer period.

At all the investigated temperatures, glycerol was detected by $^{13}C$ MAS NMR (see Paper IV). At above-zero temperatures the glycerol was detected earlier in the incubation period but glycerol signals decreased at the end of the incubation period. However, at -4°C, the glycerol produced accounted for a relatively high proportion of the newly synthesized compounds (~17 %, SE 0.01) after incubation for 99 days, when the amount of glycerol was significantly higher in the -4°C samples compared to the unfrozen samples (t-test). The first detection of glycerol at -4°C coincided with the observed increase in $^{13}CO_2$ production at the start of phase 2 (Figure 10). Simultaneously, an increase in the synthesis of membrane phospholipids was detected by $^{13}C$ MAS NMR, showing that the microbial population could grow at -4°C (the identification of phospholipids is discussed in Paper IV), as indicated in Figure 10. Glycerol

is best known as an anti-freeze medium, but it can also be used as a substrate for certain microorganisms (Panikov & Sizova, 2007; Breezee et al., 2004). The high level of glycerol found at the end of the incubation period at -4°C could inhibit intracellular cell-damage by freezing, and hence increase the unfrozen water content and affect microbial activity and $CO_2$ production in frozen soils. Thus, glycerol production and accumulation at low temperature may represent a positive feedback mechanism for microbial activity and $CO_2$ production from frozen soils.

In conclusion, I have shown that besides the expected biogenic $^{13}CO_2$ production, several metabolites were synthesized at sub-zero temperatures. In particular, the observed synthesis of membrane phospholipids demonstrates that both catabolic and anabolic processes can proceed in frozen boreal forest soils (Paper IV).

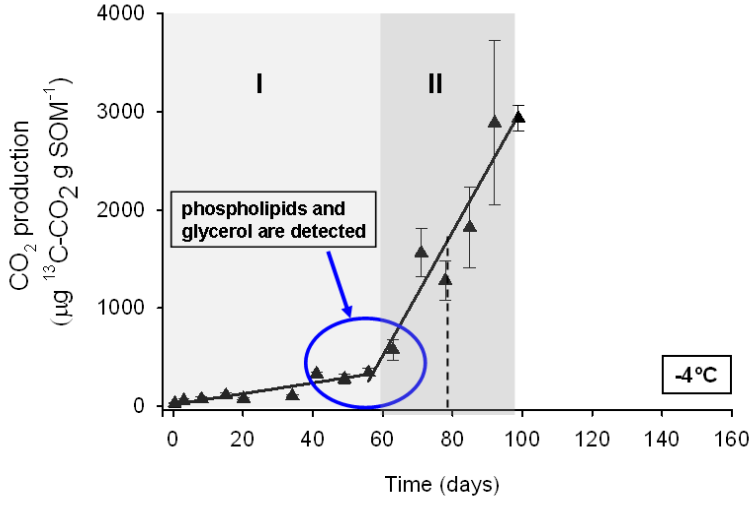



*Figure 10.* Accumulation of $^{13}CO_2$ from the incubated boreal forest soil at -4°C presented as µg $^{13}C$ g $SOM^{-1}$ as a function of time (days). Grey shaded areas indicate the different phases (I and II) identified during the incubation and the lines represent the linear regressions used to derive the $^{13}CO_2$ production rates. The first detection of phospholipids and glycerol (by NMR) coincided with the increase in $^{13}CO_2$ production at ~day 60. The dotted line illustrates the time when 50% of the glucose was left at -4°C.

At -9°C glycerol signals and weak signals in the protein and phospholipid regions were detected after 160 days, but they could not be quantified due to low signal-to-noise levels (Paper IV). However, their occurrences indicate that the microbial population was capable of utilizing glucose as a C carbon source, albeit after a lag phase of several months.

Further, the temperature of our samples was decreased more rapidly to -9°C than is likely in nature. Therefore, under natural conditions microorganisms would have more favourable conditions for adaptation than in our experiment, possibly resulting in a shorter lag-phase at -9°C. In a wider context, both the -4°C and -9°C results may have implications for the responses of soil microbes in high-latitude systems to a temperature increase. Such responses under optimal conditions may be faster than estimates obtained from short-term incubations, since threshold values for C turnover, similar to the switch from phase 1 to phase 2 observed in our study, could possibly exist in seasonally or permanently frozen soils.

### 3.3.2 Changes in C allocation between catabolic and anabolic processes across the -4°C to +9°C temperature range

Significant levels of both catabolic processes ($CO_2$ production) and anabolic processes (synthesis of phospholipids and other $^{13}C$ compounds) were detected across the -4°C to +9°C temperature range. At the times when 50% glucose had been consumed (Table 2), we observed no significant differences (t-test) between the determined CUE values and growth indexes across this temperature range (Table 2, and Paper IV). Thus, no clear differences in C allocation patterns between +9°C to -4°C were observed, although the rates were slower in the frozen soil. This conflicts with the general view of low temperature soil microbial processes; that a shift in metabolism occurs as the soil freezes and substrate diffusion becomes limited (Schimel *et al.*, 2007; Schimel & Mikan, 2005; Nedwell, 1999). However, if we had confined our investigation to the first couple of weeks, we would have drawn similar conclusions. Thus, this study importantly shows that the soil microorganisms can utilize substrates and grow as the soil freezes.

Table 2
Carbon use efficiency (CUE) and growth indices when 50% glucose was left at each of the investigated temperatures

| T (°C) | Time [a] (days) | CUE [b] | SE | Growth index [c] | SE |
|---|---|---|---|---|---|
| 9 | 2.3 | 7.69 | 0.47 | 0.78 | 0.39 |
| 4 | 2.4 | 3.50 | 0.54 | 0.57 | 0.02 |
| -4 | 79.5 | 6.92 | 2.08 | 1.84 | 0.74 |
| -9 | - | - | - | - | - |

a: The time when 50% of the glucose had been consumed
b: Amount (mg) of $^{13}C$ in newly synthesized compounds per mg $CO_2$ -$^{13}C$.
c: Amount (mg) of $^{13}C$ in lipids per mg $CO_2$ -$^{13}C$.

One significant difference between the frozen and unfrozen samples related to their C assimilation and growth was a shift in membrane lipid composition. The average numbers of double bounds (C=C) per chain in the investigated samples at the end of the incubations were 0.43 ± 0.04 (SE) at +9°C, 0.32 ± 0.05 (SE) at +4°C, and 0.69 ±0.05 (SE) at -4°C. These findings demonstrated that the average degree of unsaturation of the acyl chains in the membrane lipids was higher (t-test) at -4°C than at above 0°C, implying that the membrane lipids synthesized at -4°C were more suitable for maintaining fluidity at lower temperatures.

It is known that bacteria and fungi can adjust their membrane lipid composition in response to changes in temperature > 0°C, thus maintaining a lamellar liquid crystalline phase that is capable of sustaining physiological activity (Rilfors & Lindblom, 2002). Common features of responses to temperature changes include changes in the ratio of saturated to unsaturated fatty acids and in the length of the fatty acid chains in the membrane lipids (Morein *et al.*, 1996). If just one *cis* double bond is incorporated into each 16-carbon fatty acid tail of a phosphatidylcholine (16:1/16:1) the membrane melts at around -36°C (Mathews et al., 2000). This relationship in combination with the average length of the lipid chains (L) at -4°C (16.6 C atoms) and the average number of *cis* double bonds (0.69; at the end of the incubation) were used in Paper IV to estimate the lowest temperature at which a membrane consisting of this "average" constituents should remain fluid. The calculations indicated that this would be at around -24°C, at which the membrane lipids would transform into a more rigid gel state. The adjustment of cell membrane lipid composition may involve specific membrane adaptations and/or differences in lipid composition between different active species. However, the requirement for such adaptation by the microbial community could explain the observed $CO_2$ lag-phase in the frozen samples (Figure 10).

To summarise, the amounts of synthesized $^{13}C$ compounds, at the time when 50% glucose was consumed, were higher than the amounts of $^{13}CO_2$ at +9°C, +4°C and -4°C (95% t-test). These findings provide conclusive evidence that not only catabolic processes ($CO_2$ production), but also anabolic microbial processes (synthesis of phospholipids and other metabolites), proceed at sub-zero temperatures in frozen boreal forest soils. Thus, since the C allocation between $CO_2$ and phospholipids did not differ between -4°C and +9°C, I concluded that there appears to be no clear shift from growth to survival metabolism when the soil is subjected to freezing, contrary to the suggestion of Schimel *et al.* (2007).

## 3.4 Conclusions

- Both matric and osmotic potentials contribute to the water potential of unfrozen water in frozen boreal forest soils. From being low or negligible in unfrozen soil, the osmotic potential becomes a significant factor for both the water potential and unfrozen water contents of frozen boreal forest soils. The increase in the osmotic potential is due to concentration of the solutes in the water phase when the soil is subjected to freezing.
- At a given temperature, the pore sizes of frozen soil in which unfrozen water resides, are a determinant for heterotrophic $CO_2$ production from frozen boreal forest soils.
- SOM composition affects these pore sizes by physical effects on the pore size distribution and unfrozen water contents. Differences in SOM composition emanate from variations in vegetation cover, litter input and the degree of humification.
- The content of unfrozen water is a major determinant of the temperature response of $CO_2$ production in frozen soils and true biochemical $Q_{10}$-values can be estimated at < 0°C if the effect of unfrozen water is factored out. Hence, unfrozen water is a key factor for microbial activity and the $CO_2$ production temperature response of frozen soils.
- Both catabolic ($CO_2$ production) and anabolic (growth) processes can proceed in frozen soil at temperatures down to at least -4°C. The microbial allocation of glucose between $CO_2$ production and growth (phospholipids and other synthesized C compounds) is similar at +9°C, +4°C and -4°C. Thus, there is no clear shift in metabolism between +9°C and -4°C, only the rates are slower at -4°C.
- Physiological adjustments by the microbial community in frozen soil significantly increase the $CO_2$ production rate and their growth at < 0°C. Consequently, previous shorter incubation experiments under similar conditions to those applied in our study may have underestimated $CO_2$ production rates. Hence, provided that water and nutrient supplies are not limiting, $CO_2$ fluxes from high latitude soils during winter may be higher than estimated from shorter incubation experiments.

- My results demonstrate the difficulties involved in determining a minimum temperature threshold for soil microbial metabolism. As long as sufficient amounts of liquid water and substrates are present in the frozen soil, microbial activity should be sustained, provided that the microbes can tolerate the low soil water potentials required to keep water in its liquid state. Microbial activity could potentially be inhibited by any of these factors, but threshold values vary, depending on inherent soil characteristics.

## 3.5 Implications for winter biogeochemical processes

This thesis has provided new knowledge about low-temperature biogeochemical processes that should help attempts to elucidate the regulation of winter microbial processes. Previous observations related to low-temperature microbial activity in soils have been somewhat confusing in several respects. For instance, unrealistically high temperature response coefficients for $CO_2$ emissions have been obtained in some studies (e.g. Monson *et al.,* 2006), and there have been conflicting observations of increases in soil microbial biomass in cold soils during winter (Schadt *et al.*, 2003; Brooks *et al.*, 1998), together with reports suggesting that the microbial communities' potential to grow under such conditions is limited (e.g. Schimel *et al.,* 2007). My results suggest that microbial processes in frozen soils are surprisingly similar to those under unfrozen conditions. However, it is important to recognize that the hierarchy of controlling factors changes as the soil freezes. This has profound implications for the conceptualization of soil C dynamics.

The results may have implications, not only for boreal forest soils, but also for other high-latitude soil systems (e.g. tundra) and other environments where freezing occurs. Small differences in soil temperature can have major effects on the water content and water potential of frozen soils, which in turn have significant effects on microbial activity at sub-zero temperatures. Winter temperatures of the upper 40 cm of boreal forest soils have been estimated to be in the range -2°C to -4°C (Oquist & Laudon, 2008). If the winter soil temperature increased by about 1°C this would change the total water potential by ~1.2 MPa, which would have substantial implications for the water availability and microbial activity of frozen soil.

The importance of correct estimates of T response coefficients for winter soil $CO_2$ emission can be evaluated by comparing different approaches. Three different concepts for estimating winter loss of soil

carbon were used: 1) assuming that the observed T response during winter was the same as during the growing season; 2) assuming different T responses during unfrozen as compared to frozen conditions; and 3) using a dynamic T response function driven by the amount of unfrozen water in the frozen soil (i.e. Eq. 4; paper III). A spruce forest site with available data on soil T and soil respiration rates (Oquist, unpublished) was used as a model system. For specific values on liquid water retention at sub-zero temperatures data from a spruce forest site was used (sample K, Paper III; Figure 5). $Q_{10}$ of $CO_2$ production in the unfrozen soil at the model site has been estimated to 2.8 (soil temperature range 4-19°C, B. Erhagen personal communications), while $Q_{10}$ of $CO_2$ production in frozen O-horizon samples was 14.6 (representing the effect of both T and unfrozen water, Tilston et al, unpublished, temperatures -8°C to -2°C). Daily soil temperature data from the winter of 2003/2004 was then used to drive the three different winter respiration models. The winter was defined as the period when the daily air average T (1.7 m above soil surface) exceeded/was below 0°C (Ottosson Löfvenius, 2003). A bulk density of 0.73 g wet soil per $cm^{-3}$ was used to convert $CO_2$ production per unit soil mass to unit area.

Modelling the winter $CO_2$ production rates as a response to temperature (using a $Q_{10}$ of 2.8, from the vegetation period at this site), an average flux of 2.3 (0.02) g $CO_2$ $m^2$ $day^{-1}$ and a total winter $CO_2$ flux of 376 g $CO_2$ per $m^2$ were obtained. When $Q_{10}$-coefficients of 2.8 (T > 0°C) and 14.6 (T < 0°C) were used to estimate the winter $CO_2$ production rates, an average flux of 1.5 (0.06) g $CO_2$ $m^2$ $day^{-1}$ and a total winter $CO_2$ flux of 244 g $CO_2$ per $m^2$ were obtained. Using the model in Paper III together with data of unfrozen water, an average flux of 1.4 (0.03) g $CO_2$ $m^2$ $day^{-1}$ and a total winter $CO_2$ flux of 225 g $CO_2$ per $m^2$ were obtained. The estimated average rates are in the same range as measured winter fluxes of $CO_2$ emissions at similar sites (Sullivan *et al.*, 2008; Ilvesniemi *et al.*, 2005). However, the differences in fluxes between the first modelling approach and the latter ones demonstrate that the effect of unfrozen water on C mineralization is crucial for modelling correct winter C responses, since the $CO_2$ fluxes based on only temperature overestimate the total winter $CO_2$ flux with ca. 40%. Thus, if the effect of unfrozen water on $CO_2$ temperature response in frozen soils is ignored, this might results in substantial errors in modelling of soil C dynamics. However, the estimated $CO_2$ fluxes between approach 2) and 3) were similar, which strengthen the usability of the model in Paper III. Hence, unfrozen water could be used as an alternative way to model winter $Q_{10}$ values. This would, as indicated

above, give similar results as using $Q_{10}$ values determined from respiration data, which could simplify the modelling of soil C dynamics during winter.

This project has also shown that the soil microbial community can adapt to frozen conditions provided that nutrient supplies are sufficient, and subsequently both grow and strongly increase its $CO_2$ production. Even at -9°C, an increase in $CO_2$ production was observed after 160 days and glycerol was detected, indicating that the findings have relevance for other cold and frozen environments. The observed adaption of the microbial community and findings that the allocation of glucose to both $CO_2$ production and growth were similar at -4°C and > 0°C (Paper IV), may explain why soil microbial biomass has been reported to peak during late winter in some ecosystems (Schadt *et al.*, 2003; Lipson *et al.*, 2000). These results also provide indications of how environmental change may influence winter SOM mineralization, but further research aimed specifically at low temperature conditions are needed to further elucidate the sensitivities of the processes involved.

## 3.6 Further research

Further research related to what I have achieved:

- Glucose can be transferred directly through the cell membrane of microorganisms, while polymers need to be degraded before they can pass through the membrane, which requires exo-enzymes. Therefore, responses of the soil microbial community to different polymers at low temperatures need to be probed to determine if they can utilize complex C compounds to the same extent as glucose in frozen soils. The responses of the soil microbial community in frozen soil to lower (or no) substrate additions during long-term incubations also need to be investigated.

- The experimental system used in the studies needs to be extended, and used to investigate low-temperature processes in samples from other cold (e.g. permafrost) regions. This would elucidate if C stored in permafrost potentially could be released as $CO_2$ below 0°C, while the soil is frozen. This would have important implications for the conceptualisation and modelling of soil C dynamics as a result of thawing permafrost.

- A broader application and development of the approach used in the studies should facilitate the development of process-based mathematical models and attempts to improve predictions of soil C dynamics. Especially, the $CO_2$ temperature response dependence of availability of liquid water, at several $T < 0$°C, need to be considered. For making models on a landscape level, variations of unfrozen water at a larger scale need to be investigated.

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