Role of root litter on soil nitrogen transformations under winter climate change in forest ecosystems

冬季気候変動下における森林生態系の土壌窒素変換に根リターが果たす役割

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General introduction

1.1. Background

1.1.1. Nitrogen cycle in forest ecosystems

Atmosphere is composed by 78% of nitrogen (N) as N_2 form. Nitrogen is an essential nutrient but N_2 in atmosphere cannot be directly utilized for most of biota. In most of temperature forest ecosystems, N is an element to limit primary production (Vitousek and Howarth 1991). Primal pathway that atmospheric N_2 are incorporated into an ecosystem is chemical N fixing by lightning and biotic N fixing by bacteria (Schlesinger 1997). Nitrogen incorporated into the ecosystem exist as organic and also inorganic species. Ammonium and nitrate in soil are absorbed by plants as a nutrient. The returned N to soil via litter fall is decomposed and mineralized by soil microbes again. Mineralized N is partly absorbed by plants and soil microbes but the other is accumulated in soil and flow out from the system. Nitrogen movement within the ecosystem is called "internal cycle (intra-system cycle)". For example, Schlesinger (1997) reported the internal N cycle was 10–20-fold higher than the amount received from the outside system based on the data in a northern temperate forest in Hubbard Brook Experimental Forest (HBEF), north eastern USA. Schlesinger (1997) also estimated that the N supply for the plants from internal N cycle in the ecosystem is constructed by 31% of plant N reabsorption and 69% of detritus turnover (including decomposition, throughfall and stem flow), relative to the plant requirement. Likens and Bormann (1995) showed that the standing stock of above ground biomass and below ground biomass were 351 and 181 kg N ha^{-1}, and the litter release from litter fall and root litter were 54.2 and 6.2 kg N $ha \cdot year^{-1}$, respectively in a 55 year old forest in HBEF. Recent meta-analysis for the world wide data showed that the litter input (mass base) into soil from trees are composed by 41% of leaf, 11% of twig and 48% of root in the representative forest ecosystem (Freschet et al., 2013).

Nitrogen mineralization in soil is a significant process to drive N cycles and N availability for plants and microbes in a forest ecosystem. Nitrogen is transformed by soil microbes from organic N to ammonium N (called "ammonification") and the part of ammonium is transformed into nitrate (called "nitrification") (Schimel and Bennett 2004). At the same time, produced ammonium/ammonia and nitrate are immobilized by soil microbes. Various group and species of microbes are involved with these N transformations (Bottomley et al., 2012). In this study, I referred sum of ammonification and nitrification as "N mineralization".

Soil N mineralization by microbes is controlled by various environment factors (e.g., temperature, water, soil pH and oxygen content). Soil properties are generally heterogeneous in different spatial scales and biotic/abiotic factors such as soil structure, topography, litter quality and quantity and so on (e.g., forest ecosystems: Baldrian 2017, organic layer thickness: Kristensen et al., 2015, rhizosphere: Hinsinger et al., 2005, soil enzyme: Baldrian 2014). Active soil microbes are found in water film on surface of soil aggregate and soil porosity (Coleman 2008; Kirchman 2012), thus microbial activity is expected to be affected by

availability of substrate and energy sources around the micro habitat limited by diffusion. Further, since soil microbes inhabit in liquid water phase, water contents and the soluble matter affect the accessibility of those substrate for soil microbes (Voroney 2007; Kirchman 2012). Soil N mineralization rate is often correlated with soil quality and N budget in soil in plot and forest scale (e.g., lignin/N ratio: Scott and Binkley 1997; Prescott 2002). Similarly, soil carbon/nitrogen (C/N) ratio is considered as a factor for explaining soil N mineralization in forest stands. This regulation of chemical element on soil N transformations by soil microbes are called "stoichiometry". Sterner and Elser (2002) argued that ecological stoichiometry is based on the principles that chemical composition in a species is inherent, and the species tend to regulate the ratio equilibrium (homeostasis). The elemental homeostasis causes nutrient limitation for microbial activity. In general, labile carbon (C) addition promote soil microbial respiration and biomass under C limited environment, whereas labile N addition increase soil microbial biomass under N limited environment (Schimel and Weintraub 2003). However, main source of C and N supply in the natural ecosystems is litter fall which is complex constitution. Soil microbial C/N ratio is 8.6 ± 0.3 (Cleveland and Liptzin 2007) with C/N ratio of fungi and bacteria are ~5–15 and ~3–6, respectively (Strickland and Rousk 2010), whereas litter C/N of aboveground production (including leaf and other plants organs) and belowground production (root including all diameter class) are 66.2 ± 6.3 and 92–132 (aboveground: McGroddy et al., 2004, belowground: Silver and Miya 2001), respectively. The structure and function of soil microbial community adapt to alter the resources, and the microbial community influence the speed and direction of stoichiometric shifts of the resource throughout decomposition (Zechmeister-Boltenstern et al., 2015). Lower C/N ratio of soil microbial biomass than that of litter indicate that litter is not sufficient for soil microbes to meet their N balance. Net N mineralization occur after the resource elemental ratio reach to a critical value (Moore et al., 2006; Mooshammer et al., 2014; Zechmeister-Boltenstern et al., 2015). At the same time, lower litter C/N ratio cause lower N mineralization rate through decomposition (Ohta and Kumada 1978; Toda and Haibara 1999). However, the relationship between litter elemental ratio and N mineralization rate is not always clear (Austin and Vitousek 2012; Mooshammer et al., 2014), because other factors which also affect litter decomposition rate such as plant-litter structural feature (e.g., leaf mass per area: Pietsch et al., 2014, root diameter: Silver and Miya 2001) and litter C quality (e.g., lignin/N ratio: Scott and Binkley 1997; Prescott 2002; Trofymow et al., 2002) covary with nutrient contents in the litter (Zechmeister-Boltenstern et al., 2015).

Nitrogen mineralization rate generally show seasonal change. Temperature is a primal factor to affect microbial activity so that N mineralization rate during spring to autumn (plant growing season) tend to be high than that during winter in temperate forests (e.g., central Korea: Son and Lee 1997, northern Japan: Hishi et al., 2014, central Japan: Hirai et al., 2007, north eastern USA: Groffman et al., 2001b) and boreal forests in Alaska (Kielland et al., 2006). Besides, other study reported that there is no clear seasonal change of net N mineralization rate because of high microbial immobilization rate during both growing and winter season in arctic ecosystems (Schmidt et al., 1999). Since soil water is another factor influencing N cycle in forest ecosystems (Bai et al., 2013), rainfall with accompanying soil water changes are also significant control factor for soil N transformations. For example, Tokuchi et al. (2014) showed seasonal change of gross NH_4^+

production rate correlate with soil water content in central Japan. Landesman and Dighton (2010) showed that relationship between soil moisture and potential net N mineralization rate was different by season in eastern USA. In addition to the rainfall event, climate change such as warming, flooding, drought and drying-wetting cycle affect N cycle in forest ecosystems (Rennenberg et al., 2009). The co-limitation by temperature and water on drive factors of soil N transformations such as proteolytic enzyme activity (Brzostek et al., 2012) might affect response of N cycling in the forest ecosystems to climate change.

Soil N mineralization occurs not only during the growing season, but also during the dormant season. Soil N mineralization rate during winter accounted for more than 30% of the annual production in Japan (Hirai et al., 2007; Hishi et al., 2014) and accounted for from 6% to 12% in northeastern USA (Groffman et al., 2001b). Further, increase of NO_3^- export and soil N mineralization during growing season after severe winter climate imply that winter biological activity affect annual N cycling (Groffman et al., 2011; Shibata et al., 2013). The details of this topic is reviewed in following section and section 1.1.6.

1.1.2. Winter climate changes and soil freeze–thaw cycles

Winter has been regarded as dormant season of forest ecosystems because low temperature often limit plant productivity in cool-temperate and boreal region (Kreyling 2010). As ground litter and soil are covered by snow and often frozen by cold temperature, winter is regarded as severe environment for soil microbial activity. However, recent studies have suggested that soil microbes remain their activity in cold temperature in the field during winter (Schimel and Clein 1996; Lipson et al., 1999; Grofman et al., 2001a; Schmidt and Lipson 2004; Brooks et al., 2011), and laboratory experiments showed that microbial activity is remaining in liquid phase in frozen soil (e.g., metabolism kept at −4 °C: Drotz et al., 2010, microbial activity remain between 0 °C to −37 °C: Panikov et al., 2006).

Enough amount of snowpack can insulate soil from cold temperature to prevent soil freezing (Hardy et al., 2001; Zhang 2005) and maintain soil microbial activity beneath snowpack (Grogan and Jonasson 2006; Sorensen et al., 2016a). The snowpack decrease often cause soil freezing and soil freeze–thaw (Groffman et al., 2001a, b; Hardy et al., 2001). Winter climate change has potential alter snowpack depth in the north hemisphere and north eastern Asia (Hosaka et al., 2005; Park et al., 2010). The snowpack depth is predicted to decrease in most area of Japan except for mountainous in Tohoku region (Inoue and Yokoyama 1998). In addition, long-term measurement of snow pack change showed declining trend in Hokkaido, northern Japan (Shibata 2016). Thus, winter climate change could cause snowpack decrease and altering soil freeze–thaw pattern in northern Japan.

Changing soil freeze–thaw pattern can affect forest ecosystem processes (Campbell et al., 2005; Makoto et al., 2013). For example, soil freeze–thaw increase tree root injury and mortality (Tierney et al., 2001; Cleavitt et al., 2008; Gaul et al., 2008; Comerford et al., 2013) and decomposition of litter (leaf: Hobbie and Chapin 1996; Wu et al., 2010b, root: Wu et al., 2010a) and also decrease soil respiration (Bokhorst et al., 2013) and soil fauna (Sulkava and Huhta 2003; Templer et al., 2012; Bokhorst et al., 2013).

Since soil freeze–thaw alter water and heat regime in soil, microbial activity (such as soil N transformations) is also affected directly and indirectly ways. For example, freeze–thaw event damages soil microbes physiologically by osmotic pressure change (Jefferies et al., 2010; Lorv et al., 2014) and extracellular ice crystal formation (Bouvet and Ben 2003; Lorv et al., 2014), resulting to decrease microbial transformations of nutrient and organic matters (Nikrad et al., 2016). The freeze–thaw events also alter substrate quality and quantity for soil microbes, affecting microbial activity (e.g., ammonification, nitrification, denitrification and respiration). The details of this topic is reviewed in following section.

Freeze–thaw events have been studied as a component of biogeochemical "hot moment" defined as short periods of time that exhibit disproportionately high reaction rates relative to longer intervening time periods (McClain et al., 2003). For instance, runoff of dissolved organic carbon (DOC) into stream during snow melting season which was less than 30% of the year period accounted for 82% of the annual DOC leaching (Boyer et al., 2000) and thus the snow melting season was considered as hot moment (McClain et al., 2003). Similarly, pulse emission of nitrous oxide (N_2O) from soil caused by freeze–thaw event in cool-temperate region is regarded as a hot moment (Groffman et al., 2009). Hot moments often coincide with disturbances such as forest fire, erosion, typhoon and heavy rain (McClain et al., 2003; Kuzyakov and Blagodatskaya 2015).

At the same time, N concentration in stream and its flux are consequence of the various ecosystem processes in forest ecosystems in the watershed. In this meaning, N leaching in stream is can be an indicator of the hot moment for watershed N cycle. Nitrate export to streams show seasonal pattern and the peak is in early spring (Mitchell et al., 1996).The inter-annual variation of nitrate export during spring time and for annual average are considered to relate with soil frost intensity (Mitchell et al., 1996; Fitzhugh et al., 2003). Thus, transient period between winter and spring (i.e., early spring) can be regarded as a hot moment of N cycling in forest ecosystems. Similarly, since changes of soil freeze–thaw pattern by winter climate change potentially cause significant alternation of forest ecosystems (Groffman et al., 2001a; Campbell et al., 2005; Matzner and Borken 2008; Shibata 2016), the soil freeze–thaw could be a disturbance accompany hot moment.

1.1.3. Effect of soil freeze–thaw event on soil microbes

As I mentioned above, winter is low temperature so that soil microbial activity which affecting soil N transformations are expected in low. However, Brooks et al. (1998) have suggested that microbial biomass changes drastically during winter and the transient period in Niwot Ridge, Colorado located in the front range of the Rocky Mountains. Microbial biomass often increase during snow melt period, but decrease immediately after the snow melt in alpine ecosystems (Brooks et al., 1998; Lipson et al., 1999), an alpine forest ecosystem (Tan et al., 2014) and a boreal forest ecosystem (Sulkava and Huhta 2003). In addition, the soil freezing alters physical conditions by increasing osmotic pressure causing mortality of soil microbes (Schimel et al., 2007; Jefferies et al., 2010; Lorv et al., 2014) as mentioned above. Further, temperature

fluctuation between frozen and unfrozen (i.e., soil freeze–thaw cycle) could provide high stress for soil microbes (Matzner and Borken 2008; Brooks et al., 2011). Thus, soil freeze–thaw affect soil microbial composition and microbial activity (Yanai et al., 2004; Haei et al., 2011; Schmitt and Glaser 2011). The increase in soil microbial biomass during snow melting might be deeply link to microbial necromass that killed by soil freeze–thaw cycle, because the microbial body can be easy utilized as nutrition and/or energy source for survived microbes. It is observed that soil freeze–thaw killed soil microbes and the dead microbial body was utilized by survived microbes immediately in laboratory experiments (Skogland et al., 1988; DeLuca et al., 1992; Schimel and Mikan 2005). Fresh litter (leaves, twigs and roots) is also available source of substrate and energy source for microbes during winter as well as microbial necromass by soil freeze–thaw cycle. In the following section, I would review the current understanding about the role of root litter as a representative fresh litter supplying energy and nutrients for soil microbes.

1.1.4. Role of root litter and organic matter on soil N cycling

Plants supply litter (composing of dead leaves, stems and roots) to soil. Estimation of root litter production and its turnover rate are difficult compared to those of aboveground litter, because a root exists underground and is surrounding by decomposers and it is difficult to collect senesced root (Freschet et al., 2013). Although root litter accounted for over 40% of litter input from trees, the underground litter production has not been understood well compared to above litter production (Freschet et al., 2013; Hobbie 2015).

Bardgett et al. (2014) reviewed root function in ecosystem processes and showed that plant traits and nutrient characteristic of a root could affect soil C and N transformations after death of the root. Effect of root traits on soil C and N transformations are beginning to be viewed from resource economic spectrum which is a framework that classifying the plant traits along a more resource acquisitive strategy with fast growing species to a conservative resource acquisitive with slow growing species. For example, longer length root per unit mass (larger specific root length; SRL) has higher N contents and shorter root life span for 18 herbaceous species in central Argentina (Roumet et al., 2006). At the same time, from the chemical characteristic insight, Hobbie (2015) reviewed that high C/N ratio of root litter was not able to explain the variety of decomposability of root litter. Hobbie et al. (2010) reported that root litter immobilized N generally, and high initial content of N in root litter abate N immobilization. Therefore, different plant species might cause various N transformations because of its morphology, lifespan and chemical component and so on. However, the effect of plant species of root litter on soil N transformations under severe environment for microbes is still unclear.

In forest soil, nutrients for microbes are contained in microbial body, plant tissues (i.e., living root) and soil organic matter (i.e., humus, litter and dissolved organic matter) in forest soil (Brooks et al., 2011). Dissolved organic matter (DOM) is considered as an important C source for soil microbial activity (Marschner and Kalbitz 2003), and N source for N transformations by soil microbes (Schimel and Bennett 2004).

The sources of DOM to soil in forest ecosystems during autumn to winter are plant litter, root exudates, microbial necromass (Kalbitz et al., 2001). In winter, plant root litter can be one of the significant DOM sources under snowpack (Schmidt and Lipson 2004; Kielland et al., 2006). Plant root litter could supply organic N into soil (e.g., Tierney et al., 2001) as well as organic C (sugar) as reported elsewhere (Scott-Denton et al., 2006).

A snow removal experiment conducted in HBEF suggested that increased root litter caused by soil freeze–thaw event contributed to increase available N sources to microbes (Fitzhugh et al., 2001; Groffman et al., 2001a; Tierney et al., 2001). The increase in available N might be caused by increase in substrate for microbes through fragmentation of soil organic matter (Schimel and Clein 1996; Fitzhugh et al., 2001) and other organic matter such as litter (Shibata 2016) by soil freeze–thaw. These studies have suggested that plant root mortality possibly increased by extreme soil freeze–thaw (Tierney et al., 2001; Gaul et al., 2008). It was also suggested that root litter might be destructed by soil freeze−thaw and become an accessible organic matter (Shibata 2016). Increase in plant root litter caused by extreme soil freeze–thaw might cause surplus N for soil N cycles compared to that under non-frozen condition (Fitzhugh et al., 2001; Groffman et al., 2001a, b; Gaul et al., 2008). Although the root litter predicted to affect N transformations in soil, the prediction has not been verified well yet.

1.1.5. Effect of plant species on soil N transformations

Plant affects soil N transformation (Van de Krift and Berendse 2001) through its chemical quality and functional traits. In forest ecosystems, it is generally known that soil C/N ratio tend to be high under coniferous trees than that under broad-leaved trees (Fassnacht and Gower 1999; Yang and Luo 2011; Cools et al., 2014). It has been suggested that high lignin/N ratio of foliar litter causes high C/N ratio of organic soil layer that attribute character in mineral soil (Cools et al., 2014), and thus coniferous trees often create high C/N ratio in mineral soil (Gurmesa et al., 2013). These chemical characteristics also influence the microbial community in soil (Urbanová et al., 2015). Therefore, soil N mineralization is different among plant species and vegetation type. For example, it was often observed that soil N mineralization rate under coniferous trees is slower than that under broad-leaved trees in several studies (Fassnacht and Gower 1999; Staelens et al., 2012).

Although the effect of tree species and the role of underground production on the C and N cycling in forest ecosystems has been relatively investigated well (Augusto et al., 2002; Barbier et al., 2008; Prescott 2010; Vesterdal et al., 2013; Augusto et al., 2015; Hobbie 2015), the role of underground production of understory plants remain unclear compared to canopy trees. However, the contribution of understory plants on forest nutrient dynamics has been recognized in recent studies (Nilsson and Wardle 2005; Gilliam 2007; Fukuzawa et al., 2013). For example, the effect of understory vegetation on soil microbial community has been recognized (e.g., northern China; Zhao et al., 2017, southern China; Fu et al., 2015, subtropical China; Yin et al., 2016). Further, biodiversity of understory vegetation increased microbial activity and soil fauna

biomass in soil under coniferous forest in northern Canada (Eisenhauer et al., 2011). Mitchell et al. (2012) indicated that *Calluna vulgaris* under five single overstory trees affect soil microbial community directly and indirectly by competition with trees in Scotland. The effect of understory plant on soil microbial composition through root was also reported by Kong et al. (2017) which showed that *Sasa kurilensis* increased mycorrhizal fungi and proteobateria in the rhizome soil under birch forest in northern Japan. These results imply the significant role of root production by understory plants on soil microbial activities. Thus, understory plants could affect N cycling through root litter inputs.

1.1.6. Diverse responses of N transformations to soil freeze–thaw

Various results of the effect of soil freeze–thaw on soil N transformations in laboratory studies and field studies has been reported. For example, soil freeze–thaw increased N mineralization but not significantly affected to nitrification (Austnes and Vestgarden 2008; Christopher et al., 2008; Vestgarden and Austnes 2009). Besides, snow removal manipulation increased net ammonification rate (Shibata et al., 2013; Hishi et al., 2014) and reduced net nitrification rate (Fitzhugh et al., 2001; Groffman et al., 2011, Shibata et al., 2013; Hishi et al., 2014), whereas no significant effect of soil freeze–thaw to ammonification and nitrification were observed (Elliott and Henry 2009). Groffman et al. (2001b) reported that snow removal treatment increased soil NO_3^- concentrations significantly in sugar maple stands, whereas snow removal did not affect significantly soil N transformations in yellow birch stands. Similarly, Vestgarden and Austnes (2009) reported that leaching of NH_4^+, NO_3^- from soil under *Sphagnum* spp. than those under *Calluna vulgaris* and *Molinia caerulea* under same soil freeze–thaw regime. In addition, Urakawa et al. (2014) reported that response of N transformations to soil freeze–thaw was different among forest ecosystems. For this diverse response of soil N transformations, Groffman et al. (2011, 2012) hypothesized that DOC which varied among vegetation and increased by soil freeze–thaw affect soil N transformations under the influence of soil freeze–thaw.

Sobczak et al. (2003) and Goodale et al. (2005) suggested that increase in DOC reduced the NO_3^- export to streams because of increased N immobilization and denitrification in the soil and streambed. Broadly, there is positive relationship between DOC leaching into a stream and soil C/N ratio in the watershed (Aitkenhead and McDowell 2000; Konohira and Yoshioka 2005) and this is regarded as a result of N limitation in the soil by excess DOC production (Schimel and Weintraub 2003; Konohira and Yoshioka 2005). Similarly, under soil freeze–thaw conditions in winter, the interactions between DOC and nitrification have been observed. Some studies implied that increased DOC reduced nitrification and NO_3^- leaching in soil (Fitzhugh et al., 2001; Austnes and Vestgarden 2008; Shibata et al., 2013), while other studies have reported that there was no clear relationship between DOC and NO_3^- in soil (Hentschel et al., 2008, 2009; Vestgarden and Austnes 2009; Groffman et al., 2011). Vestgarden and Austnes (2009) reported that DOC and nutrient leaching (e.g., NH_4^+, NO_3^-) from soil under *Sphagnum* spp. under soil freeze–thaw was lower than those under *Calluna vulgaris* and *Molinia caerulea*. Flux of DOC from *Molinia* was higher than that form *Calluna*, and NO_3^- flux from *Calluna* was higher than other two species. Further, Urakawa et al. (2014) conducted a soil transplant

incubation experiment during winter throughout the Japanese archipelago and showed that the relationships between DOC production and NO_3^- production were varied among forest ecosystems and vegetation types. These varied effects of soil freeze–thaw on C and N dynamics among vegetation of the soil were also reported in tundra soil (Schimel and Clein 1996). Simultaneously trends of less nitrification and decreased NO_3^- concentration and increased DOC after soil freeze–thaw (e.g., Grofman et al., 2001b; Vestgarden and Austnes 2009) strongly imply that possible linkage between DOC dynamics and nitrification; DOC mobilized by soil freeze–thaw could stimulates immobilization or denitrification, in turn prevents net N changes (Groffman et al., 2011, 2012). Thus, there is clear need to analyze relationship between DOC and soil N transformations especially for NO_3^-, as a key mechanism of varied response of soil N transformations to soil freeze–thaw.

Durán et al. (2014) showed that soil freeze–thaw affect soil N transformations following growing season by observations winter climate gradient along elevation. They also reported that negative effect of soil freeze–thaw on nitrification was observed in the spring time, which is a critical period for nutrient losses in forest ecosystems because of much amount of runoff by snow melting (Boyer et al., 2000; Sebestyen et al., 2009; Campbell et al., 2014). Further, Haei et al. (2013) showed soil frost caused increase in DOC concentration in surface soil and heterotrophic respiration during summer by 7-year soil frost manipulation experiment in Sweden. Haei et al. (2013) pointed out the increase in DOC was caused by freeze-out processes (Ågren et al., 2012), soil physical disturbance (Oztas and Fayetorbay 2003), microbial lysis (Skogland et al., 1988) and fine root injuries (e.g., Tierney et al., 2001; Comerford et al., 2013). Thus, the change of DOC caused by soil freeze–thaw and plant root litter in winter could affect following N cycle in growing season.

1.2. Aim and focus of the study

Effect of soil freeze–thaw on soil N transformations involve complex pathway as argued above. In this study, I tried to clarify the effect of soil freeze–thaw caused by winter climate change on soil N transformations. Specifically, I focused on root litter as a mediator of effects of soil freeze–thaw on soil N transformations. Thus, the overarching question is “How does soil freeze–thaw alter soil N transformations through input of root litter under winter climate change?” To accomplish the purpose, I addressed following specific questions.

Q1. Does the different plant species of fine root litter affect to soil N transformations under freeze–thaw cycles caused by snowpack decrease?

Q2. How does soil N transformations under different vegetation response to amplified soil freeze–thaw events caused by snowpack decrease with fine root litter input?

In this thesis, I aimed to clarify the effect of root litter input and the soil freeze–thaw on N transformations in forest surface soil where is soil microbial activities is high. Especially, I focused on the interaction between dissolved organic matter (DOC and DON) and soil inorganic N transformations driven by soil microbes

(Fig.1-1). Regarding the Q1, I hypothesized that the plant species of root litter (oak vs. sasa dwarf bamboo) would affect soil N transformations during and immediate after soil freeze–thaw via changes in DOC and DON dynamics by root litter inputs (Fig. 1-2). This hypothesis was tested in Chapter 3. For the Q2, I hypothesized that the net N transformations soils under an oak forest and a larch forest would show different response to soil freeze–thaw cycle via different dissolved organic matter utility (Fig. 1-3).

1.3. Structure of the thesis

I approached the above-mentioned two research questions based on a field manipulation and laboratory experiments. The study site for in situ snow manipulation was located at an oak forest in eastern Hokkaido, Japan. The characteristics of the study site information was described in Chapter 2. I also conducted a preparatory experiment to choose the focal pattern of temperature fluctuation under winter climate change and the temperature change pattern using a laboratory incubation simulating soil freeze–thaw cycle caused by winter climate change in Chapter 3. In the laboratory soil incubation in the Chapter 3, I used fine root litter of oak and sasa dwarf bamboo that are dominant canopy and understory plants in the oak forest, respectively. In the Chapter 4, I conducted in situ soil incubation study using soils from the oak forest and an adjacent larch forest to address the Q2 in the oak forest with snow removal manipulation. Finally, I discussed the overarching question in Chapter 5 as a general discussion based on the findings and their synthesis of the Chapters 3 and 4.

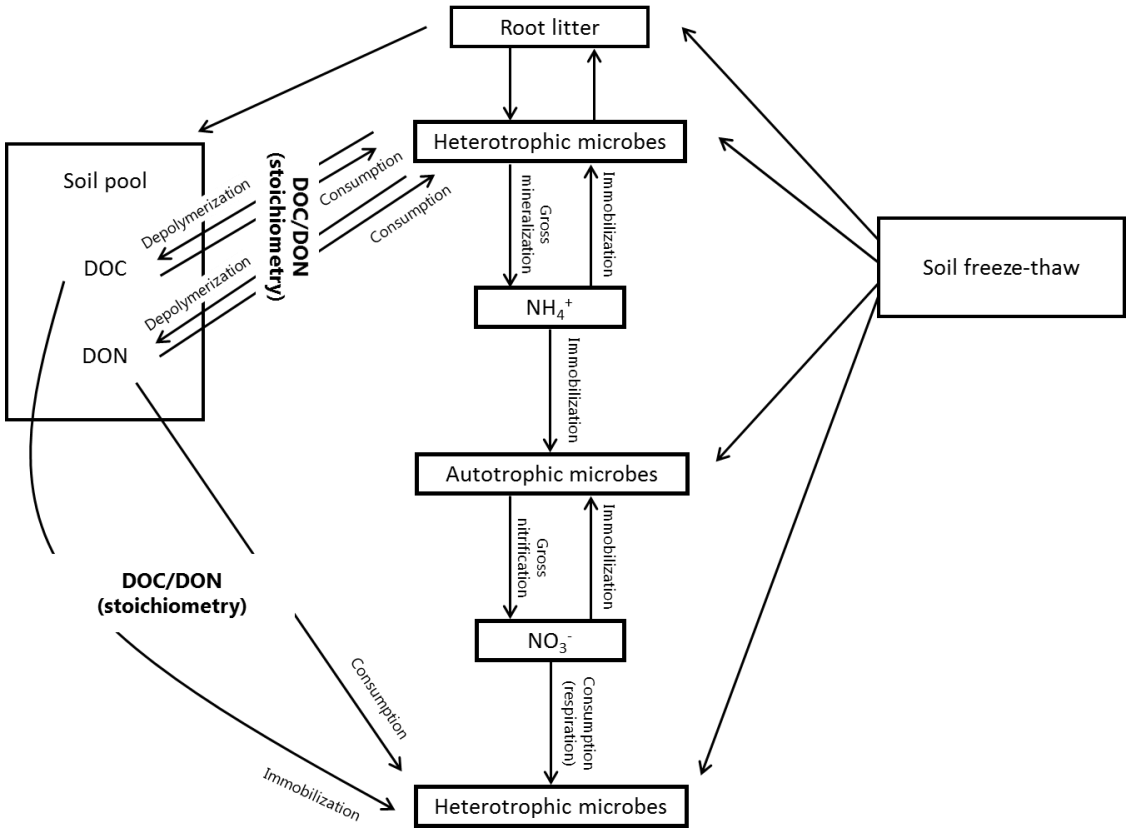


Fig. 1-1. Conceptual model of the relationship among soil N transformations, soil freeze–thaw and root litter in this thesis.

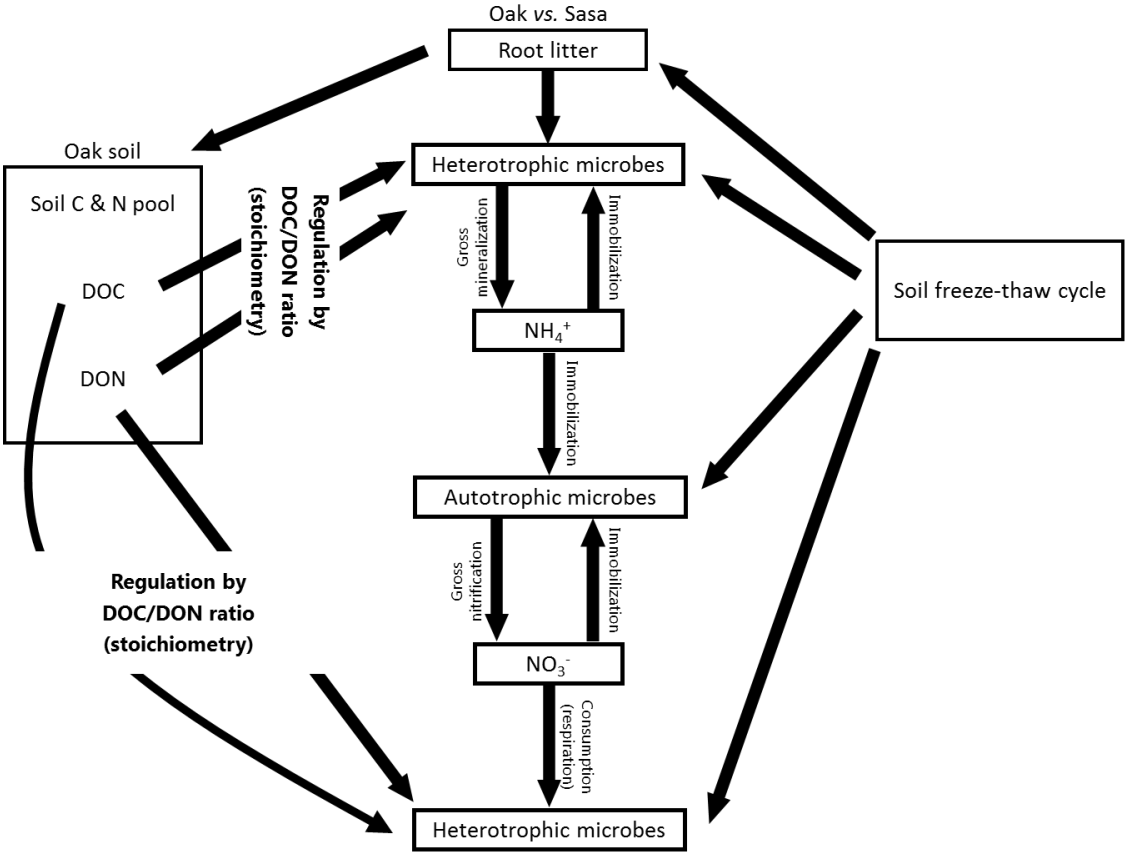


Fig. 1-2. Analytical framework of relationship among soil N transformations, soil freeze–thaw and root litter in Chapter 3.

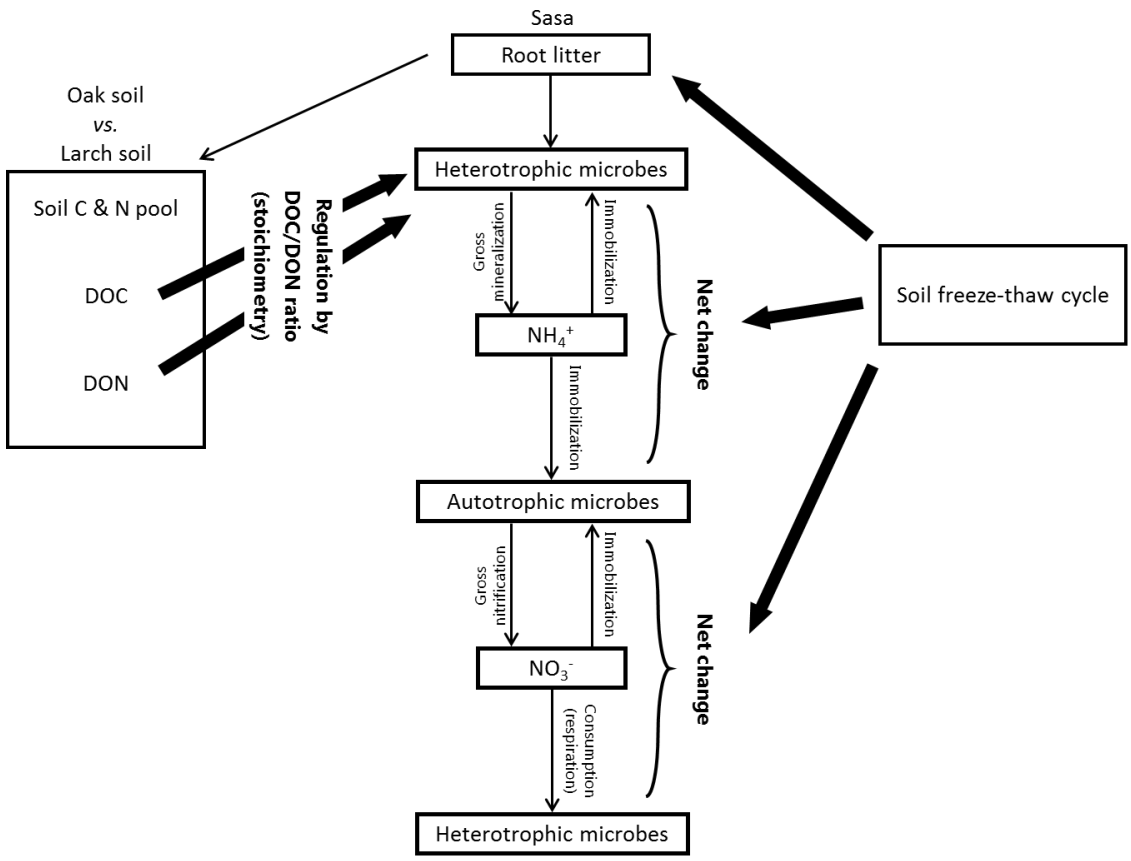


Fig. 1-3. Analytical framework of relationship among soil N transformations, soil freeze–thaw and root litter in Chapter 4. Thick arrows are focused pathways in Chapter 4.

Chapter 2
Study site and field experimental designs

In this chapter, I would describe the characteristics of study site and overall experimental designs of in situ snow removal manipulations. In addition, I describe fine root biomass and the morphology in the study site.

2.1. Site description

I conducted this study in the Shibecha branch of the Hokkaido Forest Research Station, Field Science Education and Research Center, Kyoto University (N43° 24.2', E144° 38.5', 115 m a.s.l) in eastern Hokkaido, northern Japan. According to the observed data at the meteorological station (N43° 17.5', E144° 35.2') closest to the study site, the mean annual air temperature and precipitation were 5.2 °C and 1033.7 mm, respectively, and the lowest air temperature was −25.2 °C (1981–2010). A snowpack generally persists from December until the end of April. Usually, the growing season starts in June and ends in October. Mean annual maximum snow depth was 68.7 cm (1987–2006). Soil freezing occurs from December to May. Maximum soil freezing depth varies from 35 to 50 cm in open canopy forests (Takeuchi 1981). The soil is Andosol with thin Oe and Oa layers (Nakagawa et al., 2008). The oak forest is a natural secondary forest dominated by oak (*Quercus crispula*) and dwarf bamboo (*Sasa nipponica*, hereafter referred to as Sasa). The diameters at breast height of oak are around 30 cm. The heights of oak and Sasa are about 20–25 m and 80–100 cm, respectively.

2.2. Snow removal manipulation

I conducted the field manipulation in cooperation with ReSIN-III project (research funds (25252026) of the Japan Society for the Promotion of Science; Environment Research) under the support by technical staffs of the Hokkaido forest research station, Field Science Education and Research Center, Kyoto University. I established rectangular (5 m × 12 m) snow removal plots and control plots (n = 6, respectively) in the oak forest (Fig. 2-1). The distances between adjacent plots were between 3.5 and 11.1 m. Half of the plots were placed on the eastern slope and the others were on the western slope directly opposite.

The snow removal manipulation was conducted manually with a shovel. The first snow removal was performed at the end of December, 2014. After this, snow was removed six times during winter (once a month until snow fall almost ceased in February, 2015) in the snow removal plots. Approximately 10 to 20 cm of snow cover was retained in the plots to protect understory vegetation and the soil surface from disturbance.

2.3. Fine root biomass and necromass

I collected fine root of Sasa and oak from 0–10 cm depth of mineral soil with a soil auger (inner diameter 4.4

cm) to estimate fine root (< 2 mm) biomass in control plots and snow removal plots (n = 4) before and after the snow removal. Collected soil cores were split and washed with water and through 0.25 mm mesh to collect roots. The roots were separated by species (Oak or Sasa) and live or dead (hereafter refereed as root biomass and root necromass, respectively) depend on color and elasticity. Then, the samples were dried for 48 h at 80 °C and weighted. Total C and N content of the roots were determined by CHNS/O analyzer (PE2400 II; PerkinElmer, Waltham, MA, USA). In addition, root morphology was measured in samples at August, 2014. The length and surface area of root was determined by software Win RHIZO (Regent Instruments Inc., Canada).

Specific root length (SRL) and surface area per weight (SAW) of root were larger in Sasa than oak significantly (Table 2-1). The fine root biomass of oak in top 10 cm soil ranged from 70.4 to 170 g m^{-2} that were significantly larger than that of Sasa (ranged from 24.7 to 76.7 g m^{-2}, Fig. 2-2a, b and Table 2-2). On the other hand, root necromass of oak ranged from 0.4 to 33.1 g m^{-2} that were significantly smaller than that of Sasa that ranged from 8.3 to 49.4 g m^{-2} (Fig. 2-2c, d and Table 2-2). The interaction of root species and sampling date in root necromass in the ANOVA indicated that root necromass of Sasa was larger than that of oak except for November 2015. Root necromas of Sasa was equivalent to that of Sasa root biomass during growing season (Fig.2-2). Further, C and N contents of root biomass and root necromass were significantly different between the root species but not significantly different between the manipulation (Tables 2-3 and 2-4). While oak root necromass tend to be higher in snow removal plot than that in control plot (25.8 and 21.7 g m^{-2}, respectively), Sasa root necromass was higher in control plot than that in snow removal plot (49.4 and 37.0 g m^{-2}, respectively). These results are consistent with Cleavitt et al. (2006) that reported snow removal did not affect root mortality nor root injury in northern hard wood forests in HBEF. At the same time, destructive sampling used in this study could obscure temporal variation of fine root biomass due to spatial variation between sampling time (Fukuzawa et al., 2013).

However, some previous studies reported significant effect of snow removal on root mortality. For example, Tierney et al. (2001) showed that snow removal increased plant root mortality during winter roughly 14% to 28% higher and led to an earlier peak in root production in northern hard wood forests in HBEF. Similarly, Gaul et al. (2008) reported that snow removal increased root mortality 29% higher during soil frost in a Norway spruce forest. If similar mortality (assume 30%) occurs in this site, it causes necromass equivalent to 28.2 g m^{-2} of oak and 64.2 g m^{-2} of Sasa in 0–10 cm depth soil. Using these estimated amount of root necromass and observed N concentration in root litter, I estimated the N input via root litter that was 0.17 g N m^{-2} and 0.48 g N m^{-2} from oak and Sasa respectively. Fitzhugh et al. (2001) and Tierney et al. (2001) estimated that potential excess N derived from root mortality produced by soil freeze–thaw was corresponding to 20% of the average excess N from the organic layer. These excess N is considered to be a source of N loss from a forest ecosystem via increased in soil N mineralization from decaying root necromass. My estimation above is rely on the amount of root necromass and N content and need further investigation for accurate quantification. However, this estimation suggests that root litter of oak and Sasa can be significant N source to influence N dynamics in the forest soil.

Table 2-1 Morphological character of oak and Sasa roots. Values represent means ± the standard deviations of the mean (n = 8). SRL: specific root length, SAW: surface area per weight. Asterisks denote significant difference between oak and Sasa (tested by Welch's t-test, $p < 0.05$).

Species of root	SRL (m g^{-1}) *	SAW (cm^2 g^{-1}) *
Oak	45.0 ± 21.4	383 ± 120
Sasa	83.3 ± 13.3	647 ± 75.1

Table 2-2 ANOVA results of root biomass and root necromass. ns denotes not significant ($p > 0.05$).

	Root biomass	Root necromass
Manipulation (M)	ns	ns
Sampling date (D)	ns	$p < 0.05$
Species (S)	$p < 0.05$	$p < 0.05$
Interactions:		
M × D	ns	ns
M × S	ns	ns
D × S	ns	ns
M × D × S	ns	ns

Table 2-3 Seasonal change of total carbon (TC), total nitrogen (TN) and the C/N ratio in root biomass of oak and Sasa, and the results of 3-way ANOVA. Values represent means ± the standard deviations of the mean (n = 4). ns denotes not significant (p > 0.05).

		Control		Snow removal	
Sampling date	item	Oak	Sasa	Oak	Sasa
2014/8/21	TC (%)	52.2 ± 1.71	46.9 ± 2.93	50.9 ± 1.78	47.1 ± 3.54
	TN (%)	1.36 ± 0.07	1.75 ± 0.17	1.53 ± 0.57	1.62 ± 0.31
	C/N ratio	38.3 ± 1.81	27.0 ± 3.28	38.0 ± 16.8	30.1 ± 6.74
2014/11/11	TC (%)	52.9 ± 1.87	46.9 ± 2.07	53.5 ± 1.98	45.1 ± 1.87
	TN (%)	1.41 ± 0.19	1.89 ± 0.32	1.46 ± 0.25	1.72 ± 0.45
	C/N ratio	38.3 ± 7.11	25.3 ± 3.97	37.5 ± 7.24	27.3 ± 6.39
2015/5/1	TC (%)	49.9 ± 1.20	48.1 ± 6.27	52.2 ± 2.57	47.0 ± 1.20
	TN (%)	1.37 ± 0.37	1.35 ± 0.48	1.28 ± 0.24	1.32 ± 0.35
	C/N ratio	38.8 ± 12.3	37.5 ± 7.57	42.2 ± 10.8	38.1 ± 12.7
2015/8/28	TC (%)	52.0 ± 1.18	45.3 ± 1.85	53.9 ± 2.26	47.4 ± 1.76
	TN (%)	1.38 ± 0.25	1.93 ± 0.18	1.33 ± 0.20	2.02 ± 0.15
	C/N ratio	38.8 ± 6.78	23.7 ± 3.18	41.2 ± 6.75	23.7 ± 2.81
2015/11/19	TC (%)	52.8 ± 1.03	49.0 ± 10.6	51.4 ± 2.14	46.7 ± 5.40
	TN (%)	1.30 ± 0.18	2.06 ± 0.62	1.47 ± 0.08	1.95 ± 0.34
	C/N ratio	41.4 ± 5.02	24.6 ± 4.84	35.0 ± 3.53	24.3 ± 2.70

ANOVA results

	TC	TN	C/N ratio
Manipulation (M)	ns	ns	ns
Sampling date (D)	ns	ns	p < 0.05
Species (S)	p < 0.05	p < 0.05	p < 0.05
Interactions:			
M × D	ns	ns	ns
M × S	ns	ns	ns
D × S	ns	ns	ns
M × D × S	ns	ns	ns

Table 2-4 Seasonal change of total carbon (TC), total nitrogen (TN) and the C/N ratio in root necromass of oak and Sasa, and the results of 3-way ANOVA. Values represent means ± the standard deviations of the mean (n = 4). ns denotes not significant ($p > 0.05$). Asterisks denote that all replication samples could not be analyzed because of insufficient sample amount. In this case, mean value of control plots in the same sampling date was inserted instead.

		Control		Snow removal	
Sampling date	item	Oak	Sasa	Oak	Sasa
2014/8/21	TC (%)	48.4 ± 1.78	58.6 ± 4.02	50.4 ± 2.69	53.5 ± 9.46
	TN (%)	2.33 ± 0.10	1.31 ± 0.16	2.21 ± 0.18	1.28 ± 0.63
	C/N ratio	20.9 ± 1.62	45.4 ± 8.13	23.0 ± 2.92	51.7 ± 31.4
2014/11/11	TC (%)	49.0 ± 1.71	54.1 ± 4.93	56.3 ± 8.16	54.1 ± 0.00
	TN (%)	1.28 ± 0.22	1.45 ± 0.14	1.49 ± 0.65	1.45 *
	C/N ratio	39.1 ± 7.56	37.8 ± 7.09	44.7 ± 25.6	37.3 *
2015/5/1	TC (%)	48.9 ± 4.05	47.9 ± 3.01	51.4 ± 0.88	54.8 ± 4.94
	TN (%)	1.24 ± 0.35	1.56 ± 0.21	1.37 ± 0.46	1.61 ± 0.67
	C/N ratio	42.1 ± 13.2	31.1 ± 4.56	41.2 ± 15.2	38.5 ± 15.7
2015/8/28	TC (%)	55.6 ± 5.20	47.0 ±3.18	57.9 ± 4.66	53.7 ± 2.46
	TN (%)	1.54 ± 0.27	1.40 ± 0.05	1.76 ± 0.14	1.41 ± 0.13
	C/N ratio	37.3 ± 9.39	33.7 ± 1.81	33.0 ± 2.33	38.5 ± 5.02
2015/11/19	TC (%)	44.2 ± 9.77	52.0 ±8.33	72.6 ± 21.2	49.4 ± 0.64
	TN (%)	1.70 ± 0.14	1.59 ± 0.61	1.50 ± 0.25	1.32 ± 0.22
	C/N ratio	26.1 ± 5.91	34.5 ± 6.86	51.3 ± 25.3	38.1 ± 6.51

ANOVA results

	TC	TN	C/N ratio
Manipulation (M)	ns	ns	ns
Sampling date (D)	$p < 0.05$	$p < 0.05$	ns
Species (S)	$p < 0.05$	ns	ns
Interactions:			
M × D	ns	ns	ns
M × S	ns	ns	ns
D × S	$p < 0.05$	$p < 0.05$	$p < 0.05$
M × D × S	ns	ns	ns

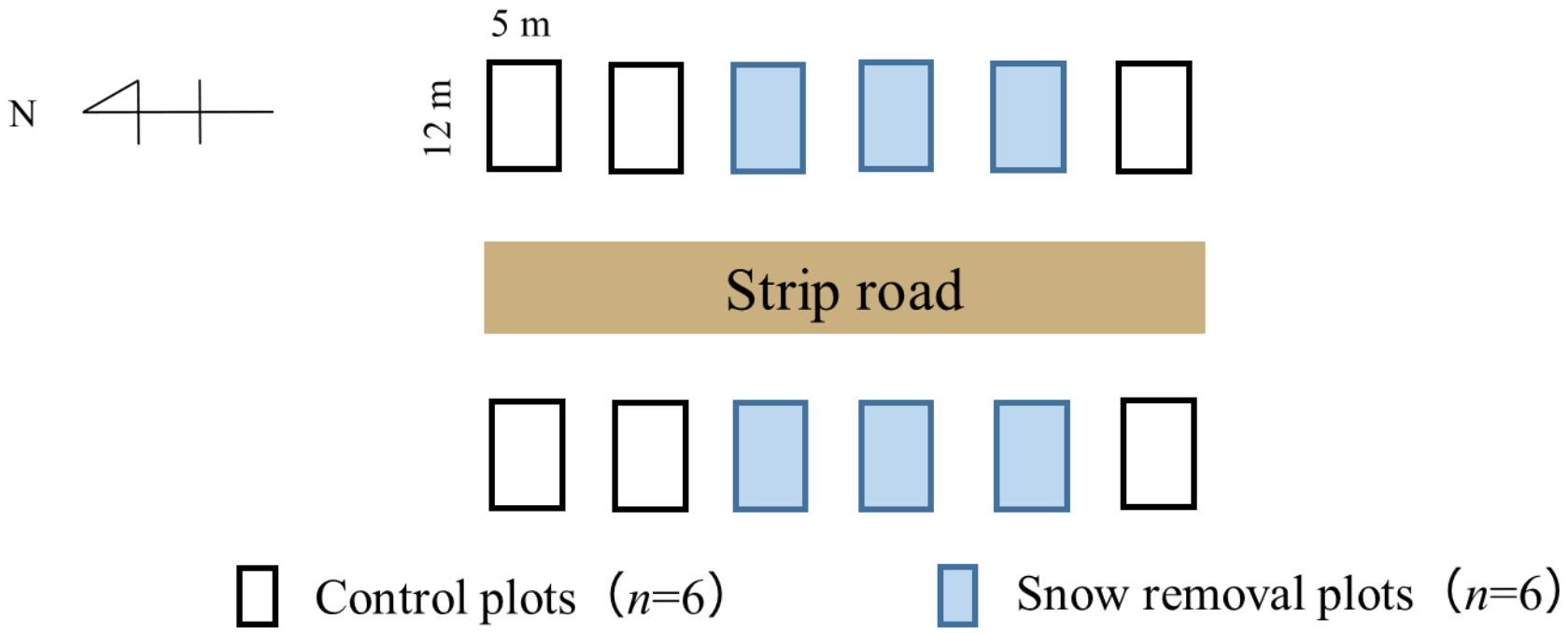


Fig. 2-1. Arrangement of study plots. Control plots and snow removal plots were selected to avoid significant differences in some soil environment factors (e.g., soil frozen depth, moisture and inorganic N concentration) between the two treatments based on measurements in the previous year. Further, the blocked arrangement gave us the practical advantage of being able to study tree physiology as another part of ReSIN-III project. The distances between adjacent plots were between 3.5 and 11.1 m because of micro-topography.

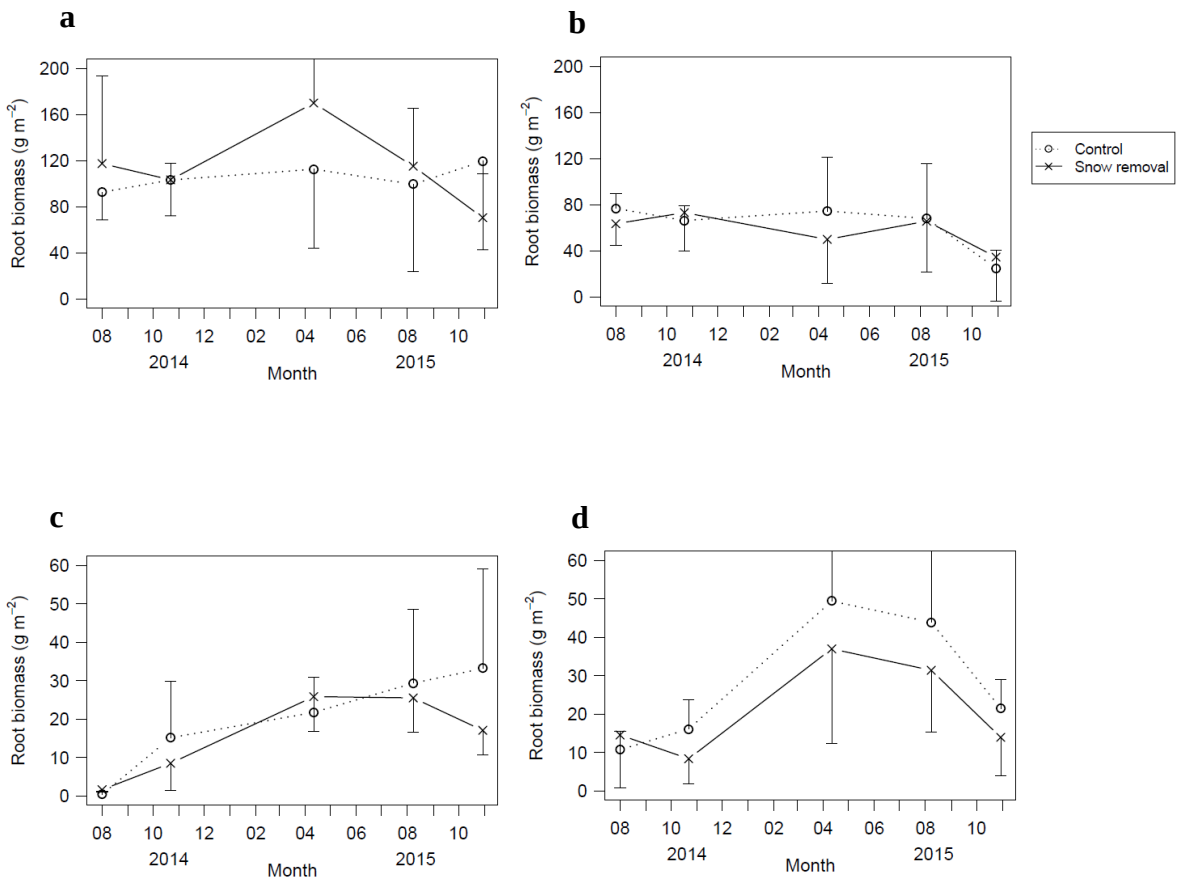


Fig. 2-2. Seasonal change of root biomass of a) oak and b) Sasa and necromass of c) oak and d) Sasa at the control and snow removal plots. Values represent means and the standard deviations of the mean (n = 4).

Chapter 3

Effect of plant species of root litter on soil nitrogen transformations under soil freeze–thaw cycle

3-1. Introduction

Microbial characteristics (activity and biomass) and the substrate properties (quality and quantity) are important factors to influence soil N transformations as described in the general introduction (Chapter 1).

A primal substrate source for soil is plant litter. Plants affect soil N transformations through its chemical quality (Van de Krift and Berendse 2001; Cools et al., 2014). However, the understanding of the effect of litter chemical quality on its decomposition and soil N transformations has been established by leaf litter (Hobbie 2015) and the understanding of species of root litter on soil N transformations is scarce compare to leaf litter (Prescott 2010; Hobbie 2015). In addition, Fukuzawa et al. (2013) reported that more than half of the below ground biomass was composed by understory plant in a northern temperate forest, suggesting the importance of the understory vegetation on soil N dynamics. Thus, evaluation of effect of root litter of understory plant species on soil N transformations is necessary as well as over story tree species.

Soil microbial biomass changes rapidly and dynamically from soil freeze–thaw (winter) to soil melting (early spring) (Sulkava and Huhta 2003; Schmidt and Lipson 2004; Tan et al., 2014). At the same time, some previous studies reported that soil freeze–thaw increased substrate leaching from leaf litter (Harris and Safford 1996) and decomposition of leaf and root litter (leaf: Hobbie and Chapin 1996; Wu et al., 2010b, root: Wu et al., 2010a). Although soil freeze–thaw could affect soil microbial biomass and also substrate quality and quantity, the temporal changes and the interaction of the soil microbes and substrates are not clarified well yet.

The various range and frequency of soil freeze–thaw provide different effects of the freeze–thaw on soil N transformations significantly (Henry 2007; Vestgarden and Austnes 2009). Although soil temperature fluctuate from sub-zero to 0 °C which soil water freeze and thaw in micro soil aggregate (Tilston et al., 2010), the understandings of the effect of this range of freeze–thaw is scarce and not well clarified yet.

I conducted laboratory incubation with root addition for two different plant species of root litter under soil freeze–thaw condition. I compared freeze–thaw conditions and constant low temperature (5 °C). In addition, I investigated the temporal changes of DOC and N transformations followed by soil freeze–thaw; i.e., immediately after and 7 days later from the soil freeze–thaw. For the precise experiment, I conducted preparatory study to choose the influential soil freeze–thaw pattern in my study site.

I hypothesized that (1) soil freeze–thaw would affect soil N transformations qualitatively and temporally (from frost to melting), (2) root litter addition would affect soil N transformations qualitatively and temporally, and (3) the different response of soil N transformations by species of root litter would be caused by difference of C/N ratio of root litter.

3.2. Material and methods

3.2.1. Preparatory experiment

I set 3 candidate soil freeze–thaw regime to verify the laboratory incubation based on the soil temperature change in Christopher et al. (2008) and the snow removal experiments in this study (details are described below). For the preparatory incubation experiments, the soil and root were collected from site SCB in the GRENE project (Urakawa et al., 2015) in Shibecha Experimental Forest. In July 2013, I trenched a 50 × 50 cm quadrat and collected mineral soil at 0–10 cm depth to collect soil and fine roots (< 2 mm) of oak from the plot. The collected soil was sieved through 2 mm mesh to remove coarse gravel and coarse organic matter. The residues from the sieving were washed with water and sieved through 4.5 mm mesh to collect the fine roots. I sorted fresh fine roots that were characterized by elasticity. The roots were washed with ion-exchanged water and were kept in plastic bag at 4 °C until the incubation. The soil incubation experiment was carried out in a 100 ml glass bottle. I placed approximate 25 g of soil into the glass with four replication for each manipulation. I added 15 mg g soil^{-1} of root (approx. root biomass in the site) into the bottle and stirred well. The soil was exposed to four different temperature regimes: +5 °C to −5 °C (extreme freeze–thaw, Extreme FT), −5 °C to 0 °C (moderate freeze–thaw, Moderate FT), −5 °C constant (Frost). As a control treatment, +5 °C constant (Non frost) for 7 days with soil without root was conducted. After these treatments, all soils were incubated at +5 °C for 2 days. Soils were extracted by 100 ml of 2 M potassium chloride (KCl) with 1:10 ratio before and after incubations.

3.2.2. Site description and sampling of soil and root litter

Mineral soil were collected from Shibecha branch of the Hokkaido Forest Research Station, Field Science Education and Research Center, Kyoto University in eastern Hokkaido, northern Japan. Detail of the study site and sampling plot were described in Chapter 2.

I collected mineral soil (10 cm depth below organic layer) in late autumn to early winter (October to December in 2014) with a shovel. Each soil block was collected from adjacent each plot, so total 12 places were used for replication. After the collection, soils were sieved through 2 mm mesh to remove coarse gravel and plant residues. After the sieving, soils kept in 4 °C in a refrigerator until start of laboratory incubation.

I collected root from the plots during October to December 2014. I dug soil about 10 cm depth from below Oa layer as block using a shovel (n = 12). I picked oak and Sasa root from collected soil blocks. Fine root (< 2 mm) were dried in air until weight reach to stable. Roots were cut into 1 cm apart to remove shape effect. The collected roots were regarded as root litter.

3.2.3. Soil incubation condition

Collected soils were packed approximate 45 to 50 g into a glass bottle independently. Soil bottles were allocated to two temperature regimes randomly in laboratory incubator as described below. One of the setup

is freeze–thaw regime (FT) that was scheduled with 7 days of freeze–thaw period that temperature was varying from –5 °C to 0 °C (12 h interval) and followed by 7 days thawing period at 5 °C (n = 9). The other setup is non-frost regime (NF) that was scheduled with constant temperature at 5 °C for 14 days (n = 6), and the incubation period set to pair up with FT: former 7 days and latter 7 days. Further, soil bottles were added root litter (root) of oak and Sasa (oak: 1.5 mg g soil^{-1} and Sasa: 1.0 mg g soil^{-1}) separately, and stirred well. Added amount of oak and Sasa were aimed to equalize the amount of added N in the two treatments (Table 3-1, p = 0.053). Although surface area per weight of the root is distinct different between the species, abundant addition of oak root litter diminished the difference (Table 3-1). Root litter was added into soil as paired with non-root addition (hereafter refereed as away-root treatment) treatment by soil blocks. Bottles were capped by cover with a hole for free airflow.

3.2.4. Potential leachate from root litter by soil freeze–thaw

Root litter was exposed to the former freeze–thaw temperature regime to estimate potential nutrient supply to the soil. Approximately 150 mg root litter of oak and Sasa were packed into two types of the glass bottle, respectively (n = 3). The root leachate is expected to be affected by water environment (i.e., water contents in a root litter and soil water surrounding the root). I determined the potential root leachate as the mean of that in water filled environment and that of air filled environment, respectively. Thus, 50 ml of ultra-pure water (UPW) was added to the one bottle, while no water was added to the other bottle. The end of the freeze–thaw, incubated roots with water was placed in room temperature for about 12 h until the ice was totally thawed. Then, the bottle was shaken gently for 1 h and the solution was percolated through a glass fiber filter (GF/F, Whatman Int. Ltd., Maidstone, Kent, UK). The roots without water was put in 50 ml of UPW after the incubation and processed in the same manner of the water-added sample. As the control, root of oak and Sasa (n = 3) were stored at 4 °C with/without UPW for 7 days and then, processed in the same manner as the frozen samples. The extracted solution was stored at 4 °C until the chemical analysis.

3.2.5. Chemical analysis

I extracted ammonium (NH_4^+), nitrate (NO_3^-), total organic C and total N with 50 mL of 2 M KCl from 5 g fresh soil at day 0, 7 and 14 after the start of the incubation. The extract was shaken for 1 h and percolated through paper filter (5B, Advantec Toyo Kaisha, Ltd, Tokyo, Japan). I used the terms dissolved organic C (DOC) and dissolved organic N (DON) to refer to total dissolved organic C and N extracted by KCl, respectively. NH_4^+, NO_3^- and total dissolved nitrogen (TDN) were measured by continuous flow colorimetry (AACS-4; BLTEC Co Ltd., Osaka, Japan). DON was determined by subtraction of inorganic nitrogen (ammonium, nitrate and nitrite) from TDN. DOC was measured by TOC analyzer (TOC-VCPH, Shimadzu, Kyoto, Japan).

I calculated net ammonification (net NH_4^+ production) and nitrification (net NO_3^- production) by

subtracting the initial concentrations of NH_4^+ and NO_3^- in the soil from those in the incubated soil, respectively. Net N mineralization was calculated by subtracting the initial concentrations of inorganic N (NH_4^+ and NO_3^-) in the soil from those in the incubated soil. I calculated the nitrification ratio by dividing nitrification by the N mineralization rate. Net DOC change and DON change were calculated in the same manner as that of net NH_4^+ production. As I used paper filter to percolate the slurry, I subtracted blank (KCl through solely paper filter) from the chemical analysis data of samples.

Microbial biomass carbon (MBC) and nitrogen (MBN) in the soil at the end of the freeze–thaw and thawing period were measured by the chloroform fumigation–extraction method (Vance et al., 1987). Fresh soil (5 g) was weighed into a glass bottle and fumigated for 24 h at 25 °C with ethanol-free chloroform. The soil was extracted with 50 mL of 0.5 M K_2SO_4 by shaking for 1 h and then percolated through paper filter (5B, Advantec Toyo Kaisha, Ltd, Tokyo, Japan). I calculated MBC and MBN as the difference in TOC and TDN between fumigated and un-fumigated samples divided by a k_{EC} of 0.45 for C (Joergensen 1996) and a k_{EN} of 0.54 for N (Joergensen and Mueller 1996).

3.2.6. Gross N mineralization and N consumption rate

I measured gross NH_4^+ production rate and NO_3^- production rate of the soils at day 7 and day 14 from the start of the incubation. Nitrogen mineralization that is distinguished immobilization is “gross N mineralization” and not distinguished is “net N mineralization”. In observation of net N mineralization, production (consumption) rate are determined by difference of concentration of NH_4^+ and NO_3^- between before and after the incubation, respectively. Combined approach to estimate both net and gross N change are expected to advance understanding soil N transformations. ^{15}N isotope dilution method is a common estimation to analysis gross N mineralization and consumption (Hart et al., 1994). In this method, NH_4^+ consumption rate include NH_4^+ immobilization by microbes and nitrification. On the other hand, NO_3^- consumption rate represents NO_3^- immobilization. The ^{15}N isotope dilution method is invented by Kirkham and Bartholomew (1954) and have been established and improved by other researchers (e.g., Davidson et al., 1991; Hart et al., 1994; Isobe et al., 2011; Kuroiwa et al., 2011). Although the measurement of this method was cumbersome and expensive, recent technical advance and diffusion of instruments, it become easier and familiar.

In ^{15}N isotope dilution method, gross mineralization is estimated by difference of N budget (i.e., NH_4^+ for ammonification and NO_3^- for nitrification) and ratio of $^{15}N/^{14}N$ ratio in soil. An assumption to permit this is microbial generation time is more than 24 hour; changes of $^{15}N/^{14}N$ ratio within 24 hour can be interpreted as gross mineralization without N recycle using microbial necromass.

I added 1 mL of ^{15}N-ammonium (ammonium chloride: ^{15}N 99.9 atom%, 1 mmol L^{-1}) or ^{15}N-nitrate (sodium nitrate: ^{15}N 99.7 atom%, 1 mmol L^{-1}) into 7 g soil and stored at 4 °C. After 24 h, I extracted the soil for the initial conditions, and after 48 h from the beginning of the incubation, I extracted the soil for the final sample. Soils were extracted with 35 mL of 2 M KCl with shaking for 1 h. Soils were centrifuged at 3500

rpm × 55 g for 10 min and filtered through a glass fiber filter (GF/F, Whatman Int. Ltd., Maidstone, Kent, UK). The amounts of inorganic nitrogen and the ratios of ^{15}N atoms were measured by GC-MS (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) through the conversion of NH_4^+ and NO_3^- to nitrous oxide (Isobe et al., 2011). Calculation of gross NH_4^+ production rate, NH_4^+ consumption rate, gross NO_3^- production rate and NO_3^- consumption rate were according to Kirkham and Bartholomew (1954).

3.2.7. Data processing and statistics

2 types of net change of inorganic N, DOC and DON were calculated for FT and NF treatments, respectively. For the estimation of change in the whole incubation period, I calculated entire net production by subtracting concentrations in the initial (day 0) from those in day 14. For examining the temporal changes of effect of soil freeze–thaw (i.e., during freeze–thaw and thawing period), I calculated partial net production by subtracting concentration day 0 and day 7 from those in day 7 and day 14, respectively. The temporal change were paired up with former 7 days (difference between day 7 and day 0) and latter 7 days (difference between day 14 and day 7) of NF treatment. On the calculation of root litter added soil, the concentration of the target substance in soil without root litter was used for the initial concentrations. For the partial net production during thawing and latter period of root added soil, concentration of root added soil at day 7 was used. Further, as gross production is affected by microbial abundance and activity, I calculated specific gross production to assess the potential activity of microbe; gross NH_4^+ (NO_3^-) production rate divided by microbial biomass carbon.

The effect of temperature regime and root addition on net N transformations in the preparatory experiment were analyzed by two-way ANOVA. If the effect of temperature regime was significant, I conducted multiple comparison by Tukey's HSD. Similarly, if the interaction between the temperature regime and root addition was significant, I applied multiple comparison among temperature regimes by root treatment (Tukey's HSD). I tested the effect of temperature regimes and difference by species of root litter on potential nutrient leachate from root litter by multivariate analysis of variance (MANOVA). The estimated supply of nutrients from root litter into soil were also analyzed by the same way with the potential nutrient leachate. For the entire net change of inorganic N, DOC and DON, I applied general linear mixed model (GLMM) to evaluate the effect of the temperature regime and root treatments (away-root, root addition of oak and Sasa) with random effect of soil blocks. I applied the Wald test for the GLMM to examine the significance of each factor and their interaction. If root treatment was significant, I tested the difference within root treatments by Tukey's HSD. I applied GLMM for DOC and N transformations and microbial biomass under soil freeze–thaw regime to analyze the effects of root addition and time (random effect were set soil blocks). I judged the significance of each statistical hypothesis test by *p*-values of 0.05. All statistical analysis were conducted using R software (R Development Core Team, 2015). I used R package "lme4" for the GLMM, "car" (Fox and Weisberg, 2011) for Wald test and "multcomp" for the multiple comparison.

3.3. Results

3.3.1. Effect of temperature regime and root addition on net N transformations in the short time period

Root litter addition significantly increased net NH_4^+ production incubated at +5 °C for 2 days after all freeze–thaw treatments (15 mg added > 0mg added). NH_4^+ production rate was net consumed in Non frost treatment, and temperature regimes transfer the rate from consumption to production (Fig. 3-1a).The difference among different temperature regimes was significant with maximum at Moderate FT treatment followed by Frost treatment. Similarly, net NO_3^- production rate increased by root addition in each freeze–thaw regime (Fig.3-1b). Net NO_3^- production rate was maximum at Extreme FT treatment and high in the order of Moderate FT and Frost treatment. Consequently, net N mineralization rate increased by root addition in each treatment (Fig. 3-1c). Net N mineralization rate was maximum in Moderate FT followed by similar amount of Frost and Extreme FT, and Non-frost. Thus, I chose the Moderate FT for the most influential soil freeze–thaw regime in this study site.

3.3.2. Potential nutrient leachate from root litter

Potential nutrient leachate was significantly different by root species except for DOC (Table 3-2). DOC/DON of dissolved organic matter from oak root litter was significantly higher than that of Sasa root litter. Potential leachate of DON and NO_3^- from Sasa root litter were significantly higher than that from oak, whereas that of NH_4^+ from oak was significantly higher than that from Sasa. Similarly, estimated supply of DON and NO_3^- from Sasa root litter into the incubated soil were significantly larger than those from oak root litter, whereas that of NH_4^+ from oak was significantly higher than that from Sasa (Table 3-3). There was no significant difference of estimated supply of DOC to the incubated soil between the root treatments (Table 3-3), although total C content in oak root litter was significantly higher than that in Sasa root litter (Table 3-1).

3.3.3. Effect of soil freeze–thaw on net N mineralization rate

Initial DOC concentration in soil was significantly different among soil blocks ($p < 0.05$ MANOVA, data not shown). The random assignation of soil blocks for temperature regimes did not have bias because I allocated soil blocks for the incubation randomly.

Entire net N mineralization (difference between day 14 and day 0) was higher in freeze–thaw regime than in non-frost regime significantly (Fig. 3-2a). Root treatment affected to entire net N mineralization significantly, but the difference by root treatment was not clear among multiple comparison; oak root addition was higher than the away-root treatment ($p = 0.07$, Tukey's HSD), whereas other comparison was not significant. The excess mineralization was larger than total N of added root litter for the both species. The higher N mineralization in freeze–thaw regime than the non-frost regime was caused by higher net NH_4^+ production rate (Fig. 3-2b). Contrary to NH_4^+ production rate, net NO_3^- production rate in soil freeze–thaw regime was significantly lower than that in the non-frost regime (Fig. 3-2c). Multiple comparison by root

treatment showed no significant differences in the net NH_4^+ production rate, but showed lower net NO_3^- production rate in Sasa root addition than in oak root (p = 0.09, Tukey's HSD).

3.3.4. Temporal changes of the effect of soil freeze–thaw on net N transformations, microbial biomass and DOM production

Net NH_4^+ production rate under freeze–thaw regime was not significantly different between the species of root litter but showed significant decrease with time (Fig. 3-3a). The degree of decrease in the net NH_4^+ production rate with time was smaller in soil with oak root litter than those in other root treatments. Net NO_3^- production rate was negative during freeze–thaw period (difference between day 7 and day 0) and thawing period (difference between day 14 and day 7), except for soil with oak root in the thawing period (Fig. 3-3b). In soil with oak root, net NO_3^- production rate increased with time, while other root treatments decreased or not changed by the time. These trends of net N production rates were contradicted with those of gross N production rates in the end of the freeze–thaw period and thawing period, respectively (Table 3-4). Soil microbes which play a role to transform N did not change in temporally its biomass while changed microbial composition (microbial C/N ratio, Table 3-4), indicating that microbial community shifted with time. DON was net consumed during all period and the consumption rate was decreased with time significantly (Table 3-4). DON concentration that is a substrate for N mineralization changed by the root treatment (p = 0.06, Table 3-4). Contrary to DON, concentration of DOC was not different among root treatments, and net DOC changes rate was distinct different among the root treatments. In freeze–thaw regime, DOC was net produced in soil with Sasa root and the net DOC production rate decreased with time. On the other hand, in soil with oak root and away-root, DOC was net consumed in the freeze–thaw period, whereas DOC was net produced in the thawing period. As a result, the difference of net DOC change rate among the root treatments was not significant in the thawing period.

Microbial biomass C (MBC) and MBN were not different among the root treatments and time (Table 3-4). Microbial C/N ratio was ranged from 7.0 to 7.6, suggesting fungi dominance (Strickland and Rousk 2010) in soils under the freeze–thaw regime. This large variation of microbial C/N ratio was obtained from maximum and minimum values of soil with oak. Microbial C/N ratio was decreased significantly with time for all root treatments.

3.3.5. Temporal changes of the effect of soil freeze–thaw on gross N transformations and DOM change

Gross N transformations showed similar change with net N transformations for all soils (Table 3-4). Gross NH_4^+ production rate increased with time, but gross NO_3^- production rate unchanged temporally except for soil with oak root. Then, I calculated gross N mineralization per microbial biomass (specific gross N transformations) as an indicator of microbial activity. Specific gross NH_4^+ production rate (SGA) significantly decreased with time except for soil with oak root (Fig. 3-4a). During freeze–thaw period, SGA

of Sasa and away were larger than that of oak, whereas SGA of oak was larger than that in Sasa and away-root during thawing period. Specific gross NO_3^- production rate (SGN) showed similar trend to SGA, but the difference between root species and period were not significant (Fig. 3-4b).

3.3.6. Contribution of root addition on microbial activity and the effect on soil N transformations and microbial biomass under soil freeze–thaw regime

The root treatment showed distinct different temporal changes and response to the temperature regimes in my results, indicating that root species affect soil N transformations different way in temporal and result in distinct difference in soil N transformations. Therefore, promotion of soil N transformations and microbial biomass caused by both temperature regimes and root addition was estimated as follows.

$$R_{nf} = \frac{P_{root,nonfrost}}{P_{away\ root,non\ frost}}$$

$$R_{ft} = \frac{P_{root,freze\text{–thaw}}}{P_{away\ root,freeze\text{–thaw}}}$$

$$E_{ft} = \frac{R_{ft}}{R_{nf}}$$

The P is the subject parameters (e.g., net NH_4^+ production rate, gross NH_4^+ production rate and microbial biomass C). R_{nf} and R_{ft} are root addition effect under non-frost and freeze–thaw temperature regimes, respectively. Negative E_{ft} of the net NH_4^+ production rate and net N mineralization rate in the both root treatment reflected that net consumption in the soil inverted into net production by root addition under non-frost regime. E_{ft} was varied among root species and objects (Table 3-5). In soil with oak root litter, both net and gross N transformations and microbial biomass were inhibited except for NO_3^- consumption rate in the freeze–thaw period, whereas net NH_4^+ production rate and gross NH_4^+ production rate and NH_4^+ consumption rate were highly stimulated (more than 3-fold) in the thawing period. Contrary to this, in soil with Sasa, net and gross NH_4^+ production rate was stimulated in the freeze–thaw period whereas net NH_4^+ production rate in thawing period was inhibited.

3.4. Discussion

3.4.1. The effect of soil freeze–thaw on supply of organic matter from root litter and microbial parameters

Although the potential organic matter supply from root litter was not significantly different between the temperature regimes, the amount of DON supply from root litter was significant different between the two root species. This result support Bokhorst et al. (2010) suggesting that leaching in autumn was significant event than winter process (e.g., soil freeze–thaw) for C and N loss from fresh leaf litter. Contrary to this, Harris and Safford (1996) reported that soil freeze–thaw increased substrate leaching from leaf litter. In addition, Hobbie and Chapin (1996) showed that winter freeze–thaw increased N release from leaf litter, implying DON leaching from plant litter. In this study, I directly measured potential nutrient leachate from samples exposed with/without freeze–thaw and with/without water. However, soil is formed by complex structure remain air, solid and water. Thus, the complex and heterogeneity of structure of soil might cause variation of breaking of root litter in the incubated soil. In this study, I could not clearly separate the effect of soil freeze–thaw and leaching on DON supply, but the DOC and DON leaching from Sasa root litter under extreme freeze–thaw (+5 °C to −5 °C) was higher than that from non-frost (Supplementary material Fig. S1). This result suggested fluctuation of soil temperature potentially increased nutrients leaching.

Contrary to my expectation, the microbial biomass did not show significant temporal change in each root treatment (Table 3-4), whereas net N transformation rates and gross N transformation rates showed significant temporal changes (Fig. 3-2 and Table 3-4). These results suggested that microbial biomass was not necessarily corresponded to magnitude of elemental (i.e., C and N) transformation rate similar to findings of Lukas et al. (2013) that soil freeze–thaw increased microbial biomass derived from maize residue without changes of microbial respiration by soil freeze–thaw and the maize addition.

3.4.2. The effect of fresh root litter input on soil N transformations

Previous studies reported contrast result of added organic matter quality on soil N transformations under soil freeze–thaw. For example, Herrmann and Witter (2002) reported no differences among soils received different organic fertilizer management. On the other hand, Su et al. (2010) reported that legume which is lower C/N than straw addition with freeze–thaw increased soil NH_4^+ and NO_3^- concentration compare to straw addition. One possible reason of the variety responses between the two studies is freshness of organic matter (Herrmann and Witter 2002); Su et al. (2010) pre-incubated soils for 2 weeks at 10 °C, whereas Herrmann and Witter (2002) pre-incubated soils for 6 months at 20 °C. The longer pre-incubation period in Herrmann and Witter (2002) might increase organic matter decomposition compare to Su et al. (2010). In this study, I did not conduct pre-incubation because I aimed to clarify the effect of root litter killed by soil freeze–thaw on soil N transformations. Thus, the result of my study similar to the result of Su et al. (2010) which used more fresh litter. Thus, the result of this study can be interpreted as a result using labile organic matter (i.e., DON) from root litter and decomposition of fresh organic matter input.

3.4.3. The effect of species of root litter on soil N transformations

Although net N mineralization for entire period (difference between day 14 and day 0) was similar among the species of root litter, temporal change of net N transformations (difference between day 7 and day 0 and that between day 14 and day 7) was distinctly different by root treatment (Fig. 3-3). Sasa root addition highly increased net NH_4^+ production rate during freeze–thaw period (difference between day 7 and day 0), whereas oak root addition increased net NH_4^+ production rate during thawing period (difference between day 14 and day 7). In addition, oak root addition increased net NO_3^- production rate during thawing period, whereas soil with Sasa root and away-root were net consumed NO_3^-.

Organic matter affect soil N transformations through its quality (Schimel and Clein 1996; Su et al., 2010). In addition, the effect of plant species on soil N transformations might be intermediated by quality and quantity of litter (Van der Krift and Berendse 2001; Kooijman and Martinez-Hernandez 2009). Thus, the contrast effect of oak root and Sasa root on the temporal changes might relate to quality and also quantity of the organic matter from root. Oak root litter contain higher total C and was higher C/N ratio than Sasa root litter (Table 3-1). Large supply of DON from Sasa root litter (Tables 3-2 and 3-3) is considered to increase net NH_4^+ production rate during freeze–thaw period. In addition, the increase of net NH_4^+ production rate ceased at thawing period indicated that supplied substrate from Sasa root litter depleted immediately. On the other hand, oak root litter significantly increased net NO_3^- production rate during thawing period (Fig. 3-3). Potential leachate of NH_4^+ and C from oak root litter was larger than those from Sasa root litter (Tables 4-2 and 4-3). Accumulation of NH_4^+ could cause higher NO_3^- production during thawing period, because nitrification follows ammonification under low temperature (Cookson et al., 2002; Dalias et al., 2002). However, NH_4^+ supply from oak was not significantly different among temperature regimes (Tables 3-2 and 3-3). Therefore it was suggested that not solely NH_4^+ but also DOC and DON contributed to the higher NO_3^- production. If DOC and DON increased NO_3^- production, it might be accompanied with increase in NH_4^+ production. Net NH_4^+ production rate decreased with time, but the reduction was less in soil with oak root litter (Fig. 3-3) implying the oak root addition increased net NH_4^+ production rate during thawing period.

A possible mechanism of the increase in N transformations in soil with oak root litter at the end of the thawing period is availability of oak root litter increased by soil freeze–thaw. Oak root had higher total C and C/N ratio. Microbes require C as well as N for their nutrient. Total N in added root were similar between the two species (p = 0.053, Table 3-1), thus higher C contents in the oak root perhaps to be advantage for microbial activity. Accessibility to C can increase by facilitation of oak root during freeze–thaw period. Physical destruction of litter by soil frozen and the increase in litter decomposition by soil freeze–thaw were observed in some previous studies (Taylor and Parkinson 1988; Hobbie and Chapin 1996; Wu et al., 2010b). In addition, delayed increase of soil N mineralization in soil with oak root was consistent with suggestion by Wu et al. (2010a) that fine root decomposition of fir could be more influenced by soil freeze–thaw than that of birch (lower C/N ratio than fir). If decomposability of oak root litter was increased by soil freeze–thaw, it is expected that fungi would increase in soil. Fungi has an advantage to initial litter decomposition than bacteria, because fungous hyphae can penetrate cell wall of plant litter (de Boer et al., 2005). In my study,

microbial C/N ratio was approximately 7.63 at the end of freeze–thaw period in soil with oak root, suggested fungi dominance; C/N ratio of fungi: ~5–13, bacteria: 3–6 (Strickland and Rousk 2010). The microbial C/N ratio was tend to be larger under freeze–thaw regime than non-frost regime in all root treatments (Table 3-4). This result consistent to a report that a species of fungi was resistant to soil freeze–thaw (Yanai et al., 2004). At the same time, Jonasson and Callagham (1992) reported that tensile stress per cross-sectional area of root of dwarf shrub was larger than that of graminoids. Thus, the physical hardiness of root litter perhaps relate to the time-lagged increase of soil N mineralization in soil with oak root litter.

3.4.4. Relationship among soil microbes, soil N transformations and organic matter of added root litter under soil freeze–thaw and soil melting

Positive effect of Sasa root litter addition on soil N transformations and microbial parameters was observed in immediate after soil freeze–thaw, whereas those of oak root litter addition occurred after thawing laggardly (Table 3-5). These time-lagged responses might relate to supply of the soluble organic matter from root litter as discussed above. Response to the organic matter were varied by parameters in each root species. Response of NH_4^+ production rate tended to be higher than that of NO_3^- production. This result suggested that soluble organic matter from Sasa root litter was more significant in ammonification in the short time period. The response of microbial biomass to soil freeze–thaw and root litter addition was lower than that of NH_4^+ production (Tables 3-4 and 3-6). These results support my hypothesis that microbial activity rather than the biomass stimulate soil N transformations as Lukas et al. (2013) suggested. Further, intensity of the promotion effect (F_{ft}) tended to be high in oak than Sasa (Table 3-5). This suggests that facilitation of decomposability of root litter by soil freeze–thaw is greater in recalcitrant root than that of easily decomposable one.

Flush of soil microbial respiration by soil freeze–thaw is often short in laboratory experiment (Schimel and Clein 1996; Lucas et al., 2013). In addition, microbial composition relating to decomposition can change by soil freeze–thaw. Yanai et al. (2004) indicated that increasing decomposition potential of chitin and decreasing the potential of rice straw by soil freeze–thaw related to increase in a species of fungi, *Fusarium* germination immediately after freeze–thaw in forest soils. The experiment period of Yanai et al. (2004) was total 78 days that is longer than this study. Thus, although the rapid, pulsed promotion effect of root litter and soil freeze–thaw could cease for the short time, the difference by species of root litter can appear in the longer time period.

3.4.5. The prolonged effect of root litter on soil N transformations in forest ecosystems

I incubated soil for 2 weeks which is relative short time period. In the field condition, winter period continue almost 4 months in this study site (Christopher et al., 2008). Longer period effect of freeze–thaw on soil N transformations through root necromass is also suggested in some previous studies (Fitzhugh et al., 2001; Tierney et al., 2001; Kielland et al., 2006). Thus, short-time experiment of this study could extrapolate into

the longer time consequence. Recalcitrant root litter such as oak, might influence for a longer time than labile root litter such as Sasa, through prolonged release of labile organic matter (Kuzyakov and Blagodatskaya 2015). Input of plant necromass also could facilitate C decomposition in soil for a longer time compare to labile substrate (Kuzyakov 2010; Luo et al., 2016). High promotion effect of NH_4^+ production activity with the oak root litter suggest that oak root litter could increase N mineralization for the following time periods. Contrary to this, effect of Sasa root litter on soil N mineralization with soil freeze–thaw might be small, although the promotion effect on soil N mineralization during freeze–thaw was higher than oak root litter. It has been suggested that labile organic matter contribute to N cycling through its rapid utility for microbes (Kielland et al., 2007). Thus, labile organic N from Sasa can be an important N sink in transient season through increase in microbial activity and biomass. These diverse effects of root litter on soil N transformations might relate to variety of root litter function of decomposition and N immobilization (Hobbie et al., 2010; Hobbie 2015).

In the future winter climate change, the decomposability of oak root litter might be facilitated by soil freeze–thaw. Further, the decomposition of oak root can be promoted by Sasa root litter through immediate increase in microbial biomass during dormant season. Further, oak is canopy tree in this study site indicate the underground production is dominated by oak. Besides, Sasa is dominant understory plants in Hokkaido and contribute comparable underground production with trees (Fukuzawa et al., 2007, 2013). In the case of leaf litter, decomposition of recalcitrant litter can be stimulated by nutrient release from adjacent rapidly decaying, higher quality litter (Gartner and Cardon 2004). The higher potential DON leachate from Sasa root than that from oak root litter (Table 3-2) implies that adjacent Sasa root litter can stimulate decomposition of oak root litter. Thus, the influence of Sasa on the forest nutrient cycle could be significant under future winter climate change. Spring time is a significant nutrient loss season because large amount of runoff by snow melting (Boyer et al., 2000; Sebestyen et al., 2009; Campbell et al., 2014). Thus, increased decomposition of root litter in the spring time could enhance C and N loss from the forest ecosystems. These difference of decomposability and diverse respond to soil freeze–thaw of plant root litter perhaps reduce C and N budget in the ecosystem.

3.5. Summary

In this chapter, I conducted laboratory soil incubation to clarify the effect of plant species (oak and Sasa) of root litter on soil N transformations under soil freeze–thaw environment. I investigated the effect of root litter addition on soil N transformations under two time period of the soil freeze–thaw (freeze–thaw period followed by thawing period). Net NH_4^+ production rate and net N production rate (= sum of NH_4^+ and NO_3^-) were significant higher under soil freeze–thaw condition than under non-frost condition. Net NO_3^- production rate under soil freeze–thaw condition was significant lower than that under non-frost condition. Root addition significantly increased net NH_4^+ production rate and net N mineralization rate, while the difference by root species was not significant. The two different root species promoted net NH_4^+ production rate in different

timing. Markedly higher leaching of dissolve organic nitrogen from Sasa root was considered to increase net NH_4^+ production rate during the freeze–thaw period. Contrary to this, it was suggested that physical fragmentation of oak root by soil freeze–thaw increased net NH_4^+ production rate during thawing period. These results suggest that the fragmentation effect of soil freeze–thaw and the fragility of the root litter could alter soil N transformations during winter and snowmelt period.

Table 3-1 Total carbon and nitrogen contents, morphological characters of oak and Sasa root and amount of root input for incubation experiment. Values represent means ± the standard deviations of the mean (n = 8, except for total nitrogen of n = 6). TC: total carbon, TN: total nitrogen, SRL: specific root length, SAW: surface area per weight. Total carbon, TN and SAW of input root litter were calculated by multiplied TC and TN contents and SAW of the roots by dry weight of added root, respectively. Asterisks denote significant differences between oak and Sasa (tested by Welch's t-test, $p < 0.05$). A dagger denotes insignificant difference between oak and Sasa (tested by Welch's t-test, $p = 0.053$).

						Input root litter			
Species of root	TC (%) *	TN (%) *	C/N *	SRL (m g^{-1}) *	SAW (cm^2 g^{-1}) *	Dry weight (mg g $drysoil^{-1}$)	TC * (mgC g $drysoil^{-1}$)	TN † (mgN g $drysoil^{-1}$)	SAW (cm^2 $drysoil^{-1}$)
Oak	52.7 ± 1.10	1.25 ± 0.21	43.1 ± 6.50	45.0 ± 21.4	383 ± 120	1.5	0.79 ± 0.017	0.019 ± 0.0031	574 ± 180
Sasa	44.8 ± 0.91	2.16 ± 0.16	20.9 ± 1.56	83.3 ± 13.3	647 ± 75.1	1.0	0.45 ± 0.0091	0.022 ± 0.0016	647 ± 75.1

Table 3-2 Dissolved organic carbon (DOC), dissolved organic nitrogen (DON), ammonium (NH_4^+) and nitrate (NO_3^-) from treated root litter and results of multivariate analysis of variance (MANOVA). Values represent means ± the standard deviations of the mean (n = 3). ns denotes not significant ($p > 0.05$).

Temperature regime	Species of root litter	DOC (mgC g^{-1})	DON (mgN g^{-1})	NH_4^+ (mgN g^{-1})	NO_3^- (mgN g^{-1})	DOC/DON
Non-frost	Oak	23.1 ± 16.5	0.63 ± 0.30	0.22 ± 0.09	0.005 ± 0.004	33.0 ± 11.4
	Sasa	27.6 ± 16.3	5.00 ± 2.63	0.13 ± 0.06	0.60 ± 0.12	5.36 ± 0.69
Freeze–thaw	Oak	13.2 ± 6.29	0.49 ± 0.20	0.18 ± 0.04	0.004 ± 0.001	26.4 ± 3.83
	Sasa	25.2 ± 13.6	4.90 ± 2.73	0.10 ± 0.04	0.63 ± 0.10	5.17 ± 0.39
	Temperature regime (T)	ns	ns	ns	ns	ns
	Species of root litter (R)	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
	Interaction T × R	ns	ns	ns	ns	ns

Table 3-3 Estimated dissolved organic carbon (DOC), dissolved organic nitrogen (DON), ammonium (NH_4^+) and nitrate (NO_3^-) from added root litter and results of ANOVA. Values represent means ± the standard deviations of the mean (n = 3). ns denotes not significant ($p > 0.05$).

Temperature regime	Species of root litter	DOC (mgC added root^{-1} kg soil^{-1})	DON (mgN added root^{-1} kg soil^{-1})	NH_4^+ (mgN added root^{-1} g soil^{-1})	NO_3^- (mgN added root^{-1} g soil^{-1})
Non-frost	Oak	34.7 ± 24.7	0.94 ± 0.46	0.32 ± 0.13	0.008 ± 0.006
	Sasa	27.6 ± 16.3	5.00 ± 2.63	0.13 ± 0.06	0.60 ± 0.12
Freeze–thaw	Oak	19.7 ± 9.43	0.73 ± 0.30	0.26 ± 0.06	0.006 ± 0.002
	Sasa	25.2 ± 13.6	4.90 ± 2.73	0.099 ± 0.04	0.63 ± 0.10
Temperature regime (T)		ns	ns	ns	ns
Species of root litter (R)		ns	$p < 0.05$	$p < 0.05$	$p < 0.05$
Interaction T × R		ns	ns	ns	ns

Table 3-4 Temporal changes of soil C and N transformations and microbial parameters and results of ANOVA under freeze–thaw regime. Values represent means ± the standard deviations of the mean. DOC: dissolved organic carbon, DON: dissolved organic nitrogen, TC: total carbon, MBC: microbial biomass carbon, MBN: microbial biomass nitrogen. ns denotes not significant ($p > 0.05$).

Item	Root treatment	Non-frost		Freeze–thaw			
		Former	Latter	Freeze–thaw	Thawing		
DON concentration ($mgN\ kg^{-1}$)	Away	31.8 ± 6.70	28.7 ± 4.50	25.0 ± 5.62	25.6 ± 5.54	Root treatment (R)	$p = 0.06$
	Oak	37.4 ± 5.56	30.1 ± 5.70	29.7 ± 5.69	26.2 ± 6.25	Period (P)	ns
	Sasa	28.8 ± 4.48	31.3 ± 2.56	22.8 ± 4.94	22.5 ± 4.46	Interaction R × P	ns
DOC concentration ($mgC\ kg^{-1}$)	Away	222 ± 42.4	211 ± 31.7	204 ± 47.4	205 ± 57.6	Root treatment (R)	ns
	Oak	253 ± 37.1	209 ± 25.8	211 ± 49.6	211 ± 58.5	Period (P)	ns
	Sasa	208 ± 32.6	225 ± 37.0	206 ± 50.8	206 ± 58.9	Interaction R × P	ns
Net DON change rate ($mgN\ kg^{-1}\ day^{-1}$)	Away	−0.11 ± 2.22	−0.45 ± 0.95	−0.93 ± 0.80	0.09 ± 0.70	Root treatment (R)	ns
	Oak	1.81 ± 0.55	−1.05 ± 0.78	−0.88 ± 0.81	−0.51 ± 0.66	Period (P)	$p < 0.05$
	Sasa	−1.67 ± 1.54	0.36 ± 0.31	−0.61 ± 0.58	−0.04 ± 0.52	Interaction R × P	ns
Net DOC change rate ($mgC\ kg^{-1}\ day^{-1}$)	Away	−1.61 ± 7.43	−1.62 ± 4.33	−1.30 ± 6.53	0.09 ± 2.60	Root treatment (R)	ns
	Oak	5.68 ± 3.94	−6.17 ± 2.55	−4.88 ± 6.17	0.01 ± 3.74	Period (P)	ns
	Sasa	−6.61 ± 2.28	2.53 ± 1.20	3.50 ± 1.84	0.001 ± 1.96	Interaction R × P	$p < 0.05$
DOC/DON ratio	Away	7.02 ± 0.58	7.45 ± 1.17	8.45 ± 2.40	8.07 ± 1.77	Root treatment (R)	ns
	Oak	6.78 ± 0.53	7.18 ± 1.54	7.30 ± 2.31	8.22 ± 2.36	Period (P)	ns
	Sasa	7.26 ± 0.91	7.18 ± 0.86	9.03 ± 0.92	9.14 ± 1.64	Interaction R × P	ns
Gross NH_4^+ production rate ($mgN\ kg^{-1}\ day^{-1}$)	Away	0.79 ± 0.46	1.78 ± 1.01	2.12 ± 0.70	1.18 ± 0.69	Root treatment (R)	ns
	Oak	0.39 ± 0.41	1.88 ± 1.28	1.84 ± 1.01	2.06 ± 0.80	Period (P)	$p < 0.05$
	Sasa	1.33 ± 1.25	2.36 ± 0.76	2.33 ± 1.46	1.67 ± 1.05	Interaction R × P	ns
Gross NO_3^- production rate ($mgN\ kg^{-1}\ day^{-1}$)	Away	0.57 ± 0.41	0.57 ± 0.29	0.42 ± 0.47	0.44 ± 0.23	Root treatment (R)	ns
	Oak	0.68 ± 0.59	0.79 ± 0.24	0.16 ± 0.20	0.57 ± 0.39	Period (P)	ns
	Sasa	0.73 ± 0.38	0.54 ± 0.39	0.41 ± 0.44	0.36 ± 0.26	Interaction R × P	$p < 0.05$
NH_4^+ consumption rate ($mgN\ kg^{-1}\ day^{-1}$)	Away	1.78 ± 1.48	1.16 ± 1.33	2.30 ± 2.53	3.00 ± 3.47	Root treatment (R)	ns
	Oak	2.64 ± 3.29	1.09 ± 0.98	1.29 ± 1.22	2.91 ± 3.23	Period (P)	ns
	Sasa	1.95 ± 2.75	0.38 ± 0.59	1.12 ± 1.53	1.20 ± 1.13	Interaction R × P	ns
NO_3^- consumption rate ($mgN\ kg^{-1}\ day^{-1}$)	Away	0.32 ± 0.36	0.50 ± 1.19	0.40 ± 0.59	0.52 ± 0.60	Root treatment (R)	ns
	Oak	0.41 ± 0.71	0.64 ± 1.38	0.41 ± 0.72	1.46 ± 1.70	Period (P)	ns
	Sasa	0.13 ± 0.16	0.32 ± 0.41	1.12 ± 0.59	0.09 ± 0.15	Interaction R × P	$p < 0.05$

MBC (mgC kg^{-1})	Away	1474 ± 588	1884 ± 312	1853 ± 540	1823 ± 668	Root treatment (R)	ns
	Oak	1231 ± 239	1754 ± 326	2176 ± 423	2076 ± 670	Period (P)	ns
	Sasa	1890 ± 262	2067 ± 307	1655 ± 570	1813 ± 668	Interaction R × P	ns
MBN (mgN kg^{-1})	Away	232 ± 41.5	276 ± 46.2	255 ± 75.6	256 ± 80.0	Root treatment (R)	ns
	Oak	261 ± 44.9	287 ± 47.4	287 ± 61.5	291 ± 74.9	Period (P)	ns
	Sasa	243 ± 37.3	274 ± 22.4	233 ± 80.0	253 ± 74.2	Interaction R × P	ns
Microbial C/N ratio	Away	6.27 ± 2.08	6.84 ± 0.68	7.31 ± 0.57	7.06 ± 0.73	Root treatment (R)	ns
	Oak	4.75 ± 0.84	6.09 ± 0.29	7.63 ± 0.53	7.01 ± 0.66	Period (P)	$p < 0.05$
	Sasa	7.79 ± 0.45	7.54 ± 0.74	7.19 ± 0.65	7.07 ± 0.80	Interaction R × P	ns

Table 3-5 Effect of root addition and soil freeze–thaw (E_{ft}) during freeze–thaw and thawing period. MBC: microbial biomass carbon, MBN: microbial biomass nitrogen.

Root species	Oak		Sasa	
Period	Freeze–thaw	Thawing	Freeze–thaw	Thawing
Net NH_4^+ production rate	0.80	−9.09	−6.19	−0.28
Net NO_3^- production rate	0.82	0.89	0.13	0.68
Net N production rate	0.85	1.28	−1.07	0.40
MBC	0.74	1.24	1.05	0.94
MBN	0.86	1.22	1.01	0.97
Gross NH_4^+ production rate	0.91	3.34	1.20	1.14
Gross NO_3^- production rate	0.66	0.77	0.33	0.67
NH_4^+ consumption rate	0.65	6.61	0.59	0.59
NO_3^- consumption rate	1.38	0.71	6.59	1.95

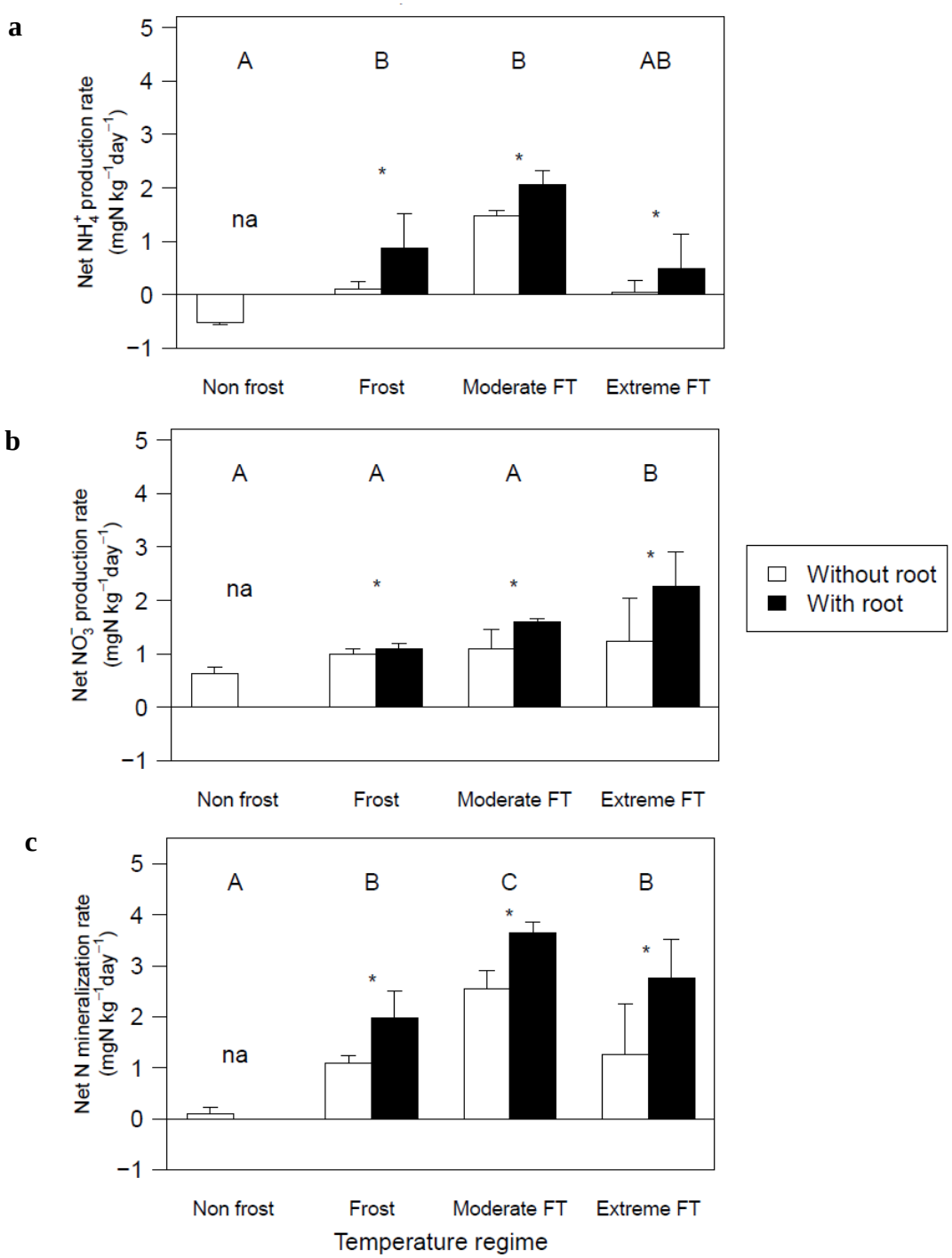
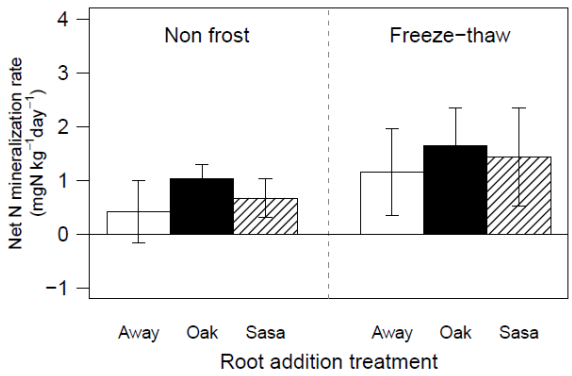


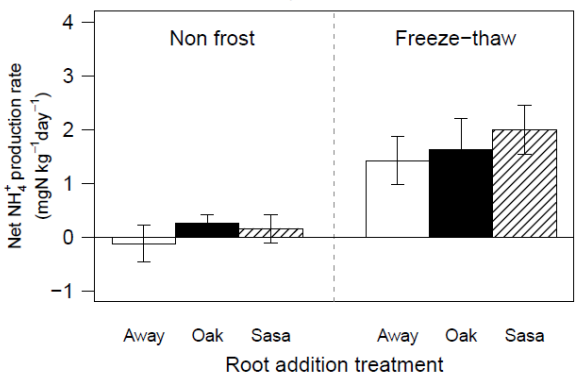
Fig. 3-1. Effect of temperature regimes on a) net NH_4^+ production rate, b) net NO_3^- production rate and c) net N mineralization rate in 2 day after thawing at 5 °C. Values represent means and the standard deviations of the mean (n = 4). White bars represent soil without root litter and filled bars represent soil with root litter. Asterisks denote significant differences with root addition (p < 0.05). Different upper cases indicate significant difference among temperature regimes (tested by Tukey's HSD, p < 0.05). na denotes not available.

a



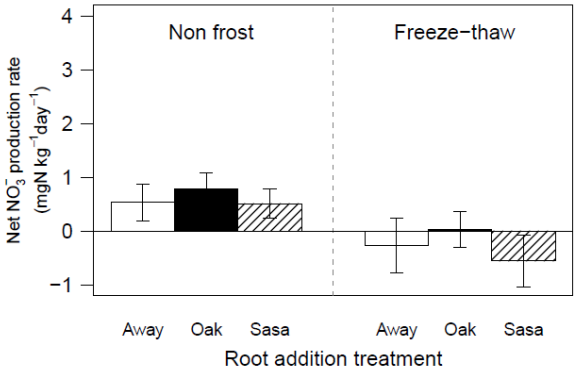
Result of ANOVA of net N mineralization rate	
Root treatment (R)	$p < 0.05$
Freeze–thaw regime (FT)	$p < 0.05$
Interaction R × FT	ns

b



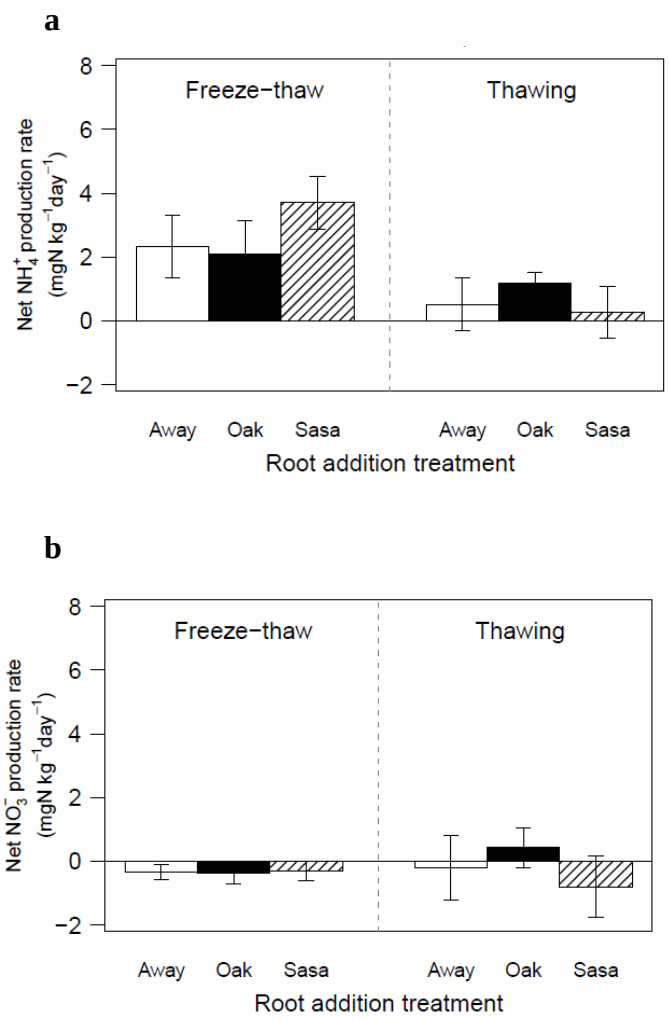
Result of ANOVA of net NH_4^+ production rate	
Root treatment (R)	$p < 0.05$
Freeze–thaw regime (FT)	$p < 0.05$
Interaction R × FT	ns

c



Result of ANOVA of net NO_3^- production rate	
Root treatment (R)	$p < 0.05$
Freeze–thaw regime (FT)	$p < 0.05$
Interaction R × FT	ns

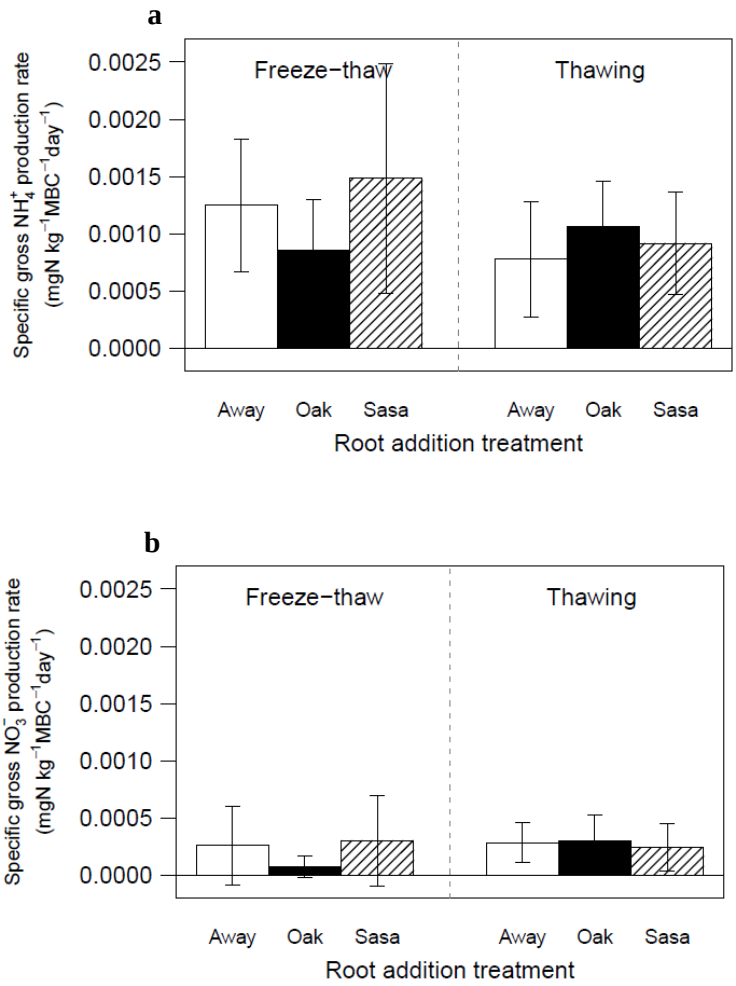
Fig.3-2. Entire net N transformations (difference between day 14 and day 0) of a) N mineralization (NH_4^+ + NO_3^-) rate, b) NH_4^+ production rate and c) NO_3^- production rate and the results of ANOVA. Values represent means and the standard deviations of the mean (n = 6 for the non-frost treatment, n = 9 for the freeze–thaw treatment). ns denotes not significant.



Result of ANOVA of net NH_4^+ production rate	
Root treatment (R)	ns
Period (P)	$p < 0.05$
Interaction R × P	$p < 0.05$

Result of ANOVA of net NO_3^- production rate	
Root treatment (R)	ns
Period (P)	ns
Interaction R × P	$p < 0.05$

Fig.3-3. Temporal change of a) net NH_4^+ production rate and b) net NO_3^- production rate under freeze–thaw regime and the results of ANOVA. Values represent means and the standard deviations of the mean (n = 9). ns denotes not significant.



Result of ANOVA of specific gross NH_4^+ production rate	
Species of root litter (R)	ns
Period (P)	$p < 0.05$
Interaction R × P	$p < 0.05$

Result of ANOVA of specific gross NO_3^- production rate	
Species of root litter (R)	ns
Period (P)	ns
Interaction R × P	ns

Fig.3-4. Temporal changes of a) specific gross NH_4^+ production rate and b) specific gross NO_3^- production rate under freeze–thaw regime and the results of ANOVA. Values represent means and the standard deviations of the mean (n = 9). ns denotes not significant.

Chapter 4

Response of nitrogen transformations to soil freeze–thaw and root litter addition in soils under two forests

4.1. Introduction

In Chapter 3, I clarified the effect of plant species of root litter on soil N transformations based on laboratory incubation experiments. This chapter focus on the effect of vegetation type on soil. Previous studies have suggested that vegetation on soil could affect response to soil freeze–thaw and also root litter input because soil microbial composition and the function is often varied among vegetation (e.g., Šnajdr et al., 2013; Urbanová et al., 2015), and tolerance to soil freeze–thaw of soil microbial community is different by vegetation (Freppaz et al., 2007). For instance, Urakawa et al. (2014) showed that the relationships between DOC production and NO_3^- production were varied among forest ecosystems and vegetation types based on a soil transplant incubation experiment during winter through the Japanese archipelago.

In eastern Hokkaido, Japan, soil is frozen in winter because of extreme low temperature and thin snow accumulation. Japanese oak (*Quercus crispula*) is a common native species in this region (Nakagawa et al., 2008), and Japanese larch (*Larix kaempferi*) is a widely planted commercial tree (Kitaoka et al., 2009) in eastern Hokkaido. Sasa dwarf bamboo often covers the forest understory in both vegetation types. Although the dwarf bamboo (e.g., *Sasa senanensis*) consists of limited aboveground biomass in the forest, they are a significant ecosystem driver in northern Japan (Fukuzawa et al., 2007, 2013; Watanabe et al., 2013).

In this chapter, I aimed to clarify the response of soil to soil freeze–thaw and root addition on soil N transformations by field experiment. I compared soils from oak forest and larch forest which stands adjacently. Thus, the environmental factors such as annual temperature and precipitation, soil type, soil texture and topography are similar.

My research questions were: 1) Do root litter inputs change N mineralization (ammonification and nitrification) and DOM production (production of DOC and DON) in soil under an extreme soil freeze–thaw event? 2) Are those responses to the soil freeze–thaw event different between soils under different vegetation? 3) Does the altered DOC affect N mineralization?

To address the above questions, I conducted an in situ snowpack removal experiment to produce an extreme freeze–thaw environment. Further, I investigated the effect of root litter on DOM production and N mineralization by experimentally adding root litter to obtain mechanistic insight into the net N change. I used Sasa roots for the soil incubation experiment, because it is a common understory plant in the two forests.

4.2. Materials and methods

4.2.1. Site description

The field experiment was conducted in the Shibecha branch of the Hokkaido Forest Research Station, Field

Science Education and Research Center, Kyoto University. I collected soil for the experiment from adjacent Japanese oak (*Quercus crispula*) forest and Japanese larch (*Larix kaempferi*) forest stands. The oak forest is a natural secondary forest dominated by oak and *Sasa nipponica* (hereafter referred to as Sasa). The diameters at breast height of oak are around 30 cm. The heights of oak and Sasa are about 20–25 m and 80–100 cm, respectively. The larch forest is a plantation forest planted in 1959. Understory of the larch forest is covered with dense Sasa. The diameters at breast height of larch are around 30 cm and the density of larch is 330 trees/ha (2014). The heights of larch and Sasa in the larch forest are about 25 m and 80–100 cm, respectively.

4.2.2. Snow removal manipulation

The detail of the snow removal manipulation was described in Chapter 2. I measured soil temperature at depths 0 and 5 cm from the soil surface at 1 h intervals using thermal sensors (TR-51 and TR-52, T & D Corp., Matsumoto, Japan and CO-UA-001, Onset Computer, Bourne, MA) during the experiment. I also measured snowpack depth in both manipulation plots by inserting meter sticks vertically into the snow (n = 3 for each treatment). The snow depth measurement was conducted several days after snow events or at two week intervals during the experimental period.

4.2.3. Soil collection

I collected soil blocks at 0–10 cm depth from the surface of the mineral soil in the oak forest and the larch soil (n = 12, respectively) in November, 2014. The replicated soil blocks were taken from two slope aspects and each set of soil blocks were 4 m apart from each other within a slope. The soils were sieved through 2 mm mesh to remove coarse gravel and roots, and stored at 4 °C. A part of the soils were dried for 48 h at 70 °C and total C and N content determined by CHNS/O analyzer (PE2400 II; PerkinElmer, Waltham, MA, USA). In December 2014, I measured pH(H_2O) (fresh soil:distilled water = 1:2.5).

4.2.4. Root sampling

In July 2013, I trenched a 50 × 50 cm quadrat and collected mineral soil at 0–10 cm depth to collect fine roots (< 2 mm) of Sasa for the soil incubation from an oak forest (site SCB in the GRENE project, Urakawa et al., 2015) in Shibecha Experimental Forest. I used Sasa root for my experiment because it is a common understory plant in the oak forest and the larch forest. The using common understory plants for the experiment might be useful to analyze the mechanism of the effect of root litter on soil N transformations under the different vegetation. The soils were washed with water and sieved through 4.5 mm mesh to collect the fine roots. I sorted fresh fine roots that were characterized by white color with elasticity. The roots were washed with ion-exchanged water and dried in air until a stable weight was reached. The roots were then cut

into 1 cm lengths to remove the effect of root shape on their decomposition, and to allow homogeneous mixing into the soil. Part of the air-dried fine roots were dried for 48 h at 70 °C and total C and N content was analyzed as described above.

4.2.5. Soil incubation

I conducted in situ soil incubation with the buried bag method (Eno 1960). Approximately 50 g of wet soil from each of the collected soil blocks was packed into a polythene bag. Half of the collected soil blocks (n = 6) were buried in control plots, and the remaining soil blocks (n = 6) were buried in snow removal plots for each vegetation type. Soil samples were buried in wells inside from the plot edge to be adequately exposed to the snow removal treatment and the control condition. The buried plots were selected randomly. Further, each soil block was separated into two: into one part the Sasa root litter was added (1 mg g drysoil^{-1} of root litter; 0.45 ± 0.01 mg C g drysoil^{-1}, 0.013 ± 0.002 mg N g drysoil^{-1} and 33.9 ± 3.7 C/N) and stirred well, while the other part remained as the control. I added root litter on the day before the beginning of the incubation, and the soils were stored at 4 °C until the beginning of the incubation. The soil moisture was adjusted to 60% water holding capacity. Four types of soil samples (from two soil origins and with/without root) were buried at a soil depth of 5 cm in each plot on the day the first snow removal was conducted, giving 48 samples in total for the incubation. The soil bags were kept in the soil from the end of December, 2014 to May, 2015 and incubated for 126 days in the field.

4.2.6. Chemical analysis

I extracted ammonium, nitrate, total organic C and total N with 50 mL of 2 M KCl from 5 g fresh soil before and after the incubation. The extract was shaken for 1 h and percolated through paper filter (5B, Advantec Toyo Kaisha, Ltd, Tokyo, Japan). I used the terms dissolved organic C (DOC) and dissolved organic N (DON) to refer to KCl extracted total organic C and N, respectively. Ammonium, nitrate and total dissolved nitrogen (TDN) were measured by continuous flow colorimetry (AACS-4; BLTEC Co Ltd., Osaka, Japan). DON was determined by subtraction of inorganic nitrogen (ammonium, nitrate and nitrite) from TDN. When the calculated DON was negative (which occurred in one third of soils without root and in half of the soils with root from the oak forest), the detection limit of TDN concentration for the auto analyzer (AACS-4; 0.01 mg N L^{-1}) was used instead. DOC was measured by TOC analyzer (TOC-VCPH, Shimadzu, Kyoto, Japan).

I calculated net ammonification (NH_4^+ production rate) and nitrification (NO_3^- production rate) by subtracting the initial concentrations of ammonium and nitrate in the soil from those in the incubated soil, respectively. Net N mineralization was calculated by subtracting the initial concentrations of inorganic N (ammonium and nitrate) in the soil from those in the incubated soil. I calculated the nitrification ratio by dividing nitrification by the N mineralization rate. Net DOC change and DON change were calculated in the same manner as that of net NH_4^+ production rate.

Microbial biomass carbon (MBC) and nitrogen (MBN) in the soil at the end of the in situ incubation were measured by the chloroform fumigation–extraction method (Vance et al., 1987). The detail of the procedure was described in Chapter 3.

4.2.7. Gross N mineralization and N consumption rate

I measured gross NH_4^+ production rate and NO_3^- production rate of the soils at the end of the in-situ incubation. The methods details were described in Chapter 3.

4.2.8. Statistical analysis

I applied a general linear mixed model (GLMM) for soil N transformations (net change in NH_4^+ production rate, NO_3^- production rate and N mineralization and nitrification ratio), DOM transformations (net change in DOC and DON and DOC/DON ratio) and microbial parameters (MBC, MBN and microbial C/N ratio) to analyze the effects of experimental factors; snow removal manipulation, vegetation type and root addition treatment. I specified the soil blocks as a random effect to deal with the violation of independency (Zuur et al., 2009) in the root addition treatment. In addition, DOC/DON ratio showed clear variation between vegetation types. Therefore, I specified different variance of error between vegetation types in the GLMM according to Zuur et al. (2009). I applied the Wald test (hereafter referred to as ANOVA) for the GLMM to examine the significance of each factor and their interaction. I judged the significance by *p*-values of 0.05. When three-way interaction was significant, I conducted two-way ANOVA within a factor (hereafter referred to as post hoc two-way ANOVA). Further, if the interaction was significant for the two-way ANOVA, I conducted one-way ANOVA (hereafter referred to as post hoc one-way ANOVA) within a factor. I conducted Welch's *t-test* for soil properties to examine the difference between the vegetation types. All statistical analyses were conducted using R software (R Development Core Team, 2015). I used the R package "nlme" (Pinheiro et al., 2013) for GLMM, and package "car" (Fox and Weisberg, 2011) for ANOVA.

4.3. Results

4.3.1. Soil properties

The oak and the larch forest soils had similar properties (Table 4-1). Initial DON concentration was significantly higher, and DOC/DON ratio was also significantly lower in the oak forest soil (hereafter referred to as oak soil) than those in the larch forest soil (hereafter referred to as larch soil). However, pH, total C content, total N content, C/N ratio and DOC concentration were not significantly different between the two vegetation types.

4.3.2. Snow depth and soil temperature

Snow removal manipulation decreased the minimum soil temperature and increased soil freeze–thaw frequency at 0 and 5 cm depth (Fig. 4-1). Soil freeze–thaw events mainly occurred from the end of December 2014 to the end of February 2015. Snow accumulation started in December, and remained through February to May 2015, with gradual snow melting during this time. Maximum snow depth in the control plots of 130 cm was observed in December 2015. This maximum snow depth was unusually large when compared with the records for December from the closest meteorological station measured during the period 1987–2006. This unusual sufficient snow depth caused less soil freezing in the control plots (Fig. 4-1) when compared with the usual winter conditions (Christopher et al., 2008).

4.3.3. Soil N transformations

Net NH_4^+ production rates were significantly higher in snow removal plots than in control plots, while the differences with vegetation and with root addition were not significant (Fig. 4-2a). Net NO_3^- production rates were significantly higher in the oak soil than in the larch soil (Fig. 4-2b). Further, net NO_3^- production rates were significantly lower in snow removal plots than in control plots, while the differences with root addition were not significant. The two-way and three-way interactions were not significant for the NH_4^+ production rate and NO_3^- production rate (data not shown). Net N mineralization rates were significantly higher in control plots than in snow removal plots. In addition, net N mineralization rates were significantly higher in the oak soil than those in the larch soil with significant three-way interaction, while the two-way interactions were not significant (data not shown). The post hoc two-way and one-way ANOVAs indicated that differences in net N mineralization rates between vegetation types were significant except for the soil with root addition in the snow removal plots (Fig. 4-3). The difference in net N mineralization rate between vegetation types was because of the difference in net NO_3^- production rate. Meanwhile, the difference in nitrification ratios by vegetation type were not significant. The nitrification ratios in the snow removal plots were about 0.40 and were significantly lower than in the control plots, where almost all N mineralization had proceeded to nitrification (Tables 4-2 and 4-3).

4.3.4. DOM transformations

DOC increased during the in situ incubation except in the oak soil with root in the snow removal plots and the larch soil without root (Fig. 4-4a). Further, the ANOVA on net DOC change rates showed a significant effect of root addition with significant vegetation type × root addition interaction and significant three-way interaction, while interactions of snow removal × vegetation type and snow removal × root addition were not significant (data not shown). Root addition significantly affected DOC change rates, but negatively in the oak soil and positively in the larch soil (post hoc two-way ANOVA) except for the oak soil in the control plots (post hoc one-way ANOVA). DON decreased in all soils during the in situ incubation (Fig. 4-4b). The

degree of decrease in DON change rates was significantly larger in the oak soil than that in the larch soil with no significant interactions (data not shown).

The DOC/DON ratios at the end of the incubation were significantly higher in the oak soil than those in the larch soil (Tables 4-2 and 4-3). The DOC/DON ratios in soil with root were significantly higher than those in soil without root. DON concentrations at the end of the incubation were highly varied in the oak soil in control plots because many samples had extremely low concentrations that were below the detection limit of the analytical instrument (Table 4-2). These extreme low values caused extremely high DOC/DON ratios in the oak soil in the control plots. Therefore, in the oak soil, DOC/DON ratios in the control plots were significantly higher than the snow removal plots, while the difference with snow removal manipulation in the larch soil was not significant (post hoc one-way ANOVA).

4.3.5. Microbial parameters

MBC contents in the oak soil were significantly higher than those in the larch soil with significant three-way interaction (Tables 4-2 and 4-3). Results of post hoc two-way and one-way ANOVAs indicated that the difference by vegetation type for MBC was significant except for soil with roots in the snow removal plots. Similarly, MBN contents in the oak soil were significantly higher than those in the larch soil with significant three-way interaction (Tables 4-2 and 4-3). Results of post hoc two-way and one-way ANOVAs indicated that the difference by vegetation type for MBN was significant except for soil with roots in the snow removal plots. Overall, the C/N ratio of microbial biomass was 3.4–4.8 (Table 4-2), which indicates that bacteria were dominant in the microbial community composition (Strickland and Rousk 2010). The differences in microbial C/N ratio by snow removal manipulation, vegetation type and root addition were not significant (Table 4-3).

4.4. Discussion

4.4.1. The effect of snow removal manipulation and root addition on soil N transformations and microbial parameters

Snow removal manipulation clearly reduced net NO_3^- production rate (Fig. 4-2b) and nitrification ratio (Table 4-3) in the two forest soils. These results are consistent with some previous studies conducted in northeastern USA and northern Japan (Groffman et al., 2011, Shibata et al., 2013; Hishi et al., 2014). Contrary to NO_3^- production rate, net NH_4^+ production rate increased with snow removal manipulation in the both vegetation types (Fig. 4-2a). This result is consistent with previous studies conducted in northern Japan (Hishi et al., 2014; Shibata et al., 2013) and laboratory incubations with soil from southern Norway (Austnes and Vestgarden 2008; Vestgarden and Austnes 2009). NH_4^+ production might be accelerated by soil freeze–thaw through increased input of organic C and N caused by fragmentation of soil aggregates and mortality of roots and microbes (Fitzhugh et al., 2001). Although net NH_4^+ production was increased by

snow removal, snow removal reduced net NO_3^- production drastically, suggesting that microbes involved in nitrification were vulnerable to soil freeze–thaw (Wang and Bettany 1994; Austnes and Vestgarden, 2008; Urakawa et al., 2014). This is also supported by previous studies (Cookson et al., 2002; Dalias et al., 2002), which showed that nitrification occurred slowly at 4 °C and was delayed following NH_4^+ production, indicating that nitrifying microbes need longer time periods to acclimate to low temperature than ammonifying microbes.

These changes in NH_4^+ production rate and NO_3^- production rate resulted in significant differences in net N mineralization rate with vegetation type and snow removal manipulation. Root addition did not affect net NH_4^+ production rate and net NO_3^- production rate, but did affect N mineralization rate in the snow removal plots (Figs. 4-2 and 4-3). Root addition probably eliminated the difference in net N mineralization rate between the two vegetation types under extreme soil freeze–thaw conditions in the snow removal plots (Fig. 4-3). This was perhaps because root addition tended to decrease the reduction in net N mineralization rate by extreme soil freeze–thaw in the larch soil, while this decreasing trend with root addition was not observed in the oak soil. I expected changes in microbial composition (microbial C/N ratio) and microbial biomass with root addition under the extreme freeze–thaw condition in the snow removal plots, if the root addition altered the soil N transformation rates through increased available C or N for microbes. However, microbial composition was not different with root addition treatments under both snow removal manipulations (Tables 4-2 and 4-3). Coincidentally, the differences in MBC and MBN with vegetation type under extreme freeze–thaw conditions were eliminated with root addition (Tables 4-2 and 4-3, post hoc ANOVAs). These differences were perhaps caused by the slight increase in microbial biomass with snow removal in the larch soil with root addition, and the slight decrease in microbial biomass with snow removal in the oak soil with root addition (Table 4-2). Tolerance to soil freeze–thaw was varied by vegetation type and land use even in the same mountainous region (Freppaz et al., 2007). Therefore, it is possible that microbial composition in the larch soil was more tolerant to soil freeze–thaw than that in the oak soil. Further, if microbes in the larch soil were more soil freeze–thaw tolerant, they would retain the ability to decompose and utilize nutrients, specifically the available C and N derived from root litter that was fragmented by extreme soil freeze–thaw in the snow removal plots.

4.4.2. The effect of vegetation type on DOM transformations

Net DOC change rates showed contrasting responses to root addition with vegetation type (Fig. 4-4a). In the oak soil, DOC was net produced in soils without root, and root addition decreased DOC production rates in both of the manipulation plots. In contrast, DOC was net consumed in soils without root, while root addition resulted in net DOC production rates in both manipulation plots in the larch soil. Therefore, root addition appeared to increase available C in the larch soil, and decrease it in the oak soil. Coincidentally, MBC contents in the oak soil tended to be lower in soil with roots than those in soil without roots in the snow removal plots. These results suggest the possibility that the microbial community in the oak soil has less

ability to respond (through decomposition and C use) to the increase in organic matter than that in the larch soil. Microbial community composition affects the response of soil C and N dynamics to nutrient addition (Waldrop and Zak 2006 ; Su et al., 2010). Waldrop and Zak (2006) showed that the response of DOC production to nitrate amendment in soils under different vegetation was affected by soil microbial composition (fungi:bacteria ratio) and the amount of phenol oxidase. Therefore, the different responses of DOC change with vegetation to root addition are possibly related to microbial community composition and the associated soil enzymes that result in the specific microbial decomposability and utility of C and N. However, the microbial C/N ratio did not reflect differences in microbial community composition by vegetation type.

Šnajdr et al. (2013) compared fungi:bacteria ratios among some tree species planted in a post-mining area, and showed that soils under larch and alder had more bacteria than those under other tree species such as oak and spruce. However, the genetic composition of bacteria in soil under the larch was varied and differences in community composition among the tree species were not clear (Urbanová et al., 2015). Meanwhile, Wang et al. (2013) reported that soil under larch had larger bacterial biomass than soil under broad-leaved trees estimated by the PLFA method in a mountainous area of northeast China. Further investigation is necessary to clarify the relationships among microbial community composition, microbial activities and response of DOC production with organic matter addition.

4.4.3. The effect of snow removal manipulation on soil N transformations through increased root litter

Although a clear effect of root addition on DOC change rate was observed, root addition did not significantly affect net NH_4^+ production rate, net NO_3^- production rate and net DON change rate (Figs. 4-2 and 4-4). In addition, the differences in gross N transformations (gross NH_4^+ production rate, gross NO_3^- production rate and nitrate consumption rate) in the incubated soil with root addition were not significant except for the ammonium consumption rate (Tables 4-4 and 4-5).

Fitzhugh et al. (2001) and Tierney et al. (2001) estimated that potential excess N derived from root mortality produced by soil freeze–thaw corresponded to 20% of the average excess N from the organic layer. Contrary to their studies, I did not observe excess N mineralization or DON production with root addition. This difference was possibly because of methodological differences (field measurement vs. soil incubation) and plant species of added root. They mainly investigated root of broad leaved trees, while my experiment was conducted with Sasa root. Decomposition of *Sasa senanensis* culm and leaf litter are slower than that of tree leaf litter (Watanabe et al., 2013). Therefore, decomposition of Sasa root was possibly slower and to a lesser extent than that of the broad-leaved litter. In contrast, Fitzhugh et al. (2001) observed excess soil solution N in the field with snow removal manipulation that was higher than the potential excess N from root mortality. Fitzhugh et al. (2001) and Tierney et al. (2001) thought that root mortality might significantly affect soil N transformation rates, but that the effect might be relatively small in comparison to the high variation in N mineralization rates. Therefore, in my study, the effect of root litter on soil N transformation

rates and DOC dynamics was possibly significant in the two vegetation types, but the degree and the contribution of root litter might vary by plant species of root litter.

4.4.4. Interaction of microbes, DOC change and soil N transformations under extreme soil freeze–thaw

Production of available C (DOC) was increased by root addition in the larch soil under mild freeze–thaw conditions in the control plots (Fig. 4-4a). In addition, extreme soil freeze–thaw in the snow removal plots amplified the increase in available C production, and extreme soil freeze–thaw reduced net NO_3^- production rate (Fig. 4-2b). Meanwhile, in the oak soil, available C was decreased by root addition and extreme soil freeze–thaw amplified the difference with root addition; extreme soil freeze–thaw also reduced net NO_3^- production rate in the oak soil. Therefore, regardless of microbial composition and DOC change, extreme soil freeze–thaw significantly reduced NO_3^- production rate. These results are in contrast to those of Fuss et al. (2016) that observed high DOC and constant NO_3^- leaching from soil following soil freeze–thaw. In my study, root addition, rather than soil freeze–thaw affected DOC dynamics significantly in soils under the two forest types.

However, microbial biomass values were not drastically changed by snow removal manipulation and root addition (Tables 4-2 and 4-3). One possible reason for this is that microbial biomass represents total microbial biomass regardless of function, i.e., microbial biomass would not reflect specific microbes related to DOC production/consumption and nitrification/nitrate consumption. In addition, microbial biomass changed rapidly within a short period of several days; previous studies showed that microbial biomass increased at the beginning of the snow melting period but decreased immediately afterward (Brooks et al., 1998; Lipson et al., 1999; Sulkava and Huhta 2003). This response can be explained by the utilization of microbial necromass killed in the soil freeze–thaw process by surviving microbes for respiration (Skogland et al., 1988) and immobilization (DeLuca et al., 1992). In my study, the differences in nitrate consumption rate by snow removal and root addition were not significant (Tables 4-4 and 4-5). Further, ammonium consumption rate was significantly reduced by root addition. These contradictory results of gross N transformations in contrast with net N transformations might be related to the microbial nature of rapid turnover and generation. The active species in the microbial community associated with nitrification and denitrification can change drastically over time (Smith et al., 2010). Therefore, detection of the relationships among microbial biomass, DOC change and soil N transformations by microbes under soil freeze–thaw conditions might be difficult to study in the field. Laboratory studies that are able to detect rapid changes in microbial community structure and function would advance the knowledge about this topic. In addition, an experiment designed for repeated sampling in the field might be effective to determine the detailed dynamics of microbial community and functioning.

4.4.5. Over-winter effect of soil freeze–thaw on C and N cycling in forest ecosystems

In this study, I focused on the dormant seasonal effect of the soil freeze–thaw through root litter inputs. Soil freeze–thaw could increase fine root mortality followed by root compensatory regrowth in the growing season that causes a reduction in nitrification in soil (Sorensen et al., 2016b). Fine roots might be sensitive to soil freeze–thaw and thus contribute to N loss from soil (Vankoughnett and Henry 2013). Thus, analyzing root dynamics and its role during the growing season as well as the dormant season would be necessary.

Some previous studies conducted in Japan showed that soil freeze–thaw caused high N mineralization in the following growing season (Hishi et al., 2014; Urakawa et al., 2014). DOC production during winter under soil freeze–thaw conditions might be related to N mineralization in the growing season (Urakawa et al., 2014). Therefore, the expected increase in root litter (Tierney et al., 2001) and root exudates from injured roots (Comerford et al., 2013) caused by intense and frequent soil freeze–thaw conditions related to climate change will alter N dynamics in forest ecosystems. My results suggest that various responses of C and N dynamics to soil freeze–thaw and root litter input exist among different forest types. Higher annual N mineralization in artificial larch forest than that in natural broad-leaved forest including oak was observed in an area with similar climate to my site (Hishi et al., 2014), therefore higher N mineralization annually and in the growing season in the larch forest than that in the oak forest might happen under current climate conditions in my site. In larch forest, the increase in root litter might reduce annual N mineralization through increased DOC. Contrary to this, in oak forest, the increase in root litter might increase annual N mineralization through decreased DOC. These possible contradictory responses could reduce the current differences between forest types, and the alteration of winter climate change on N cycling could be offset at the landscape scale. However, Durán et al. (2016) suggested that in situ NH_4^+ production and nitrification decreased with increasing soil freeze–thaw, and thus N availability in a northern hardwood forest would be lower under future climate change. The inconsistent perspective between Durán et al. (2016) and this study might be caused by the time-scale differences: 40 years vs. one season as well as biotic and abiotic conditions (e.g., Spodosol vs. Andosol) in the studied sites. Analyzing temporal changes with multiple environmental and biological factors (e.g., N deposition; Vankoughnett and Henry 2013) should advance the understanding of the complex responses of ecosystem N cycles to winter climate change.

4.5. Summary

In this chapter, I conducted a snow removal experiment to simulate extreme soil freeze–thaw to understand the effect of soil freeze–thaw on soil N transformations in soils under two northern temperate forests (oak and larch). I also investigated the effect of root input on microbial biomass and transformations of dissolved organic carbon (DOC) and N by experimentally adding root litter to obtain mechanistic insight. Snow removal significantly reduced net NO_3^- production and N mineralization, while it increased net NH_4^+ production in both soils. Root litter addition provided contrasting effects of vegetation type on net DOC change; it significantly decreased net DOC production in the oak soil, while it was increased in the larch soil.

The contrasting responses of DOC change with vegetation were possibly related to specific microbial composition and physiology of the vegetation. Further, root addition eliminated the differences in net N mineralization and microbial biomass between the two vegetation types in the snow removal plots. These results indicated that soil freeze–thaw could reduce the differences in vegetation type on soil N dynamics through root litter input.

Table 4-1 Soil properties of the top 10 cm mineral soil. TC: total carbon content, TN: total nitrogen content, C/N: total C/N ratio, DOC: dissolved organic carbon concentration, DON: dissolved organic nitrogen concentration and DOC/DON: DOC/DON ratio. Values represent means ± standard deviations of the mean (n = 12). Asterisks denote significant differences between soil under the oak forest and under the larch forest (tested by Welch's t-test, $p < 0.05$).

Vegetation	pH (H_2O)	TC (%)	TN (%)	C/N	DOC (mg C kg^{-1})	DON (mg N kg^{-1}) *	DOC/DON *
Oak forest	5.0 ± 0.3	9.5 ± 1.0	0.67 ± 0.08	14.4 ± 0.7	231 ± 28.6	32.1 ± 3.83	7.2 ± 0.5
Larch forest	5.2 ± 0.2	9.9 ± 1.8	0.67 ± 0.11	14.7 ± 0.7	236 ± 34.8	28.9 ± 4.01	8.2 ± 0.6

Table 4-2 Nitrification ratio and dissolved organic carbon concentration (DOC), dissolved organic nitrogen concentration (DON), DOC/DON ratio (DOC/DON), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and microbial C/N ratio (Microbial C/N) in the soil at the end of the incubation. Values represent means ± standard deviations of the mean (n = 6).

	Control				Snow removal			
	Oak soil		Larch soil		Oak soil		Larch soil	
Parameters	Without Root	With Root	Without Root	With Root	Without Root	With Root	Without Root	With Root
Nitrification ratio	0.96 ± 0.29	0.99 ± 0.29	0.97 ± 0.02	1.01 ± 0.03	0.41 ± 0.39	0.58 ± 0.53	0.39 ± 0.54	0.16 ± 0.93
DOC (mg C kg^{-1})	283 ± 56.8	266 ± 30.5	232 ± 32.8	273 ± 50.9	269 ± 18.8	233 ± 20.3	218 ± 38.6	290 ± 37.0
DON (mg N kg^{-1})	7.6 ± 6.7	11.9 ± 18.1	23.0 ± 2.8	24.0 ± 2.4	12.1 ± 2.9	14.4 ± 9.5	23.1 ± 4.5	23.7 ± 3.5
DOC/DON	601 ± 912	633 ± 638	10.1 ± 1.3	11.4 ± 1.4	23.8 ± 8.5	24.2 ± 18.1	9.5 ± 0.8	12.3 ± 0.6
MBC (mg C kg^{-1})	736 ± 262	791 ± 198	500 ± 241	361 ± 147	741 ± 200	529 ± 188	437 ± 86.1	463 ± 175
MBN (mg N kg^{-1})	160 ± 57.7	183 ± 45.1	106 ± 53.3	94.9 ± 36.0	189 ± 31.5	152 ± 45.5	102 ± 27.3	113 ± 36.6
Microbial C/N	4.8 ± 2.0	4.6 ± 1.9	4.8 ± 0.8	4.1 ± 1.6	3.9 ± 0.6	3.4 ± 0.4	4.5 ± 0.9	4.1 ± 0.6

Table 4-3 Results of ANOVA for nitrification ratio and DOC/DON ratio (DOC/DON), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and microbial C/N ratio (microbial C/N) in the soil at the end of the incubation. ns denotes not significant ($p > 0.05$)

Parameters	Snow removal	Vegetation	Root	Snow removal × Vegetation	Snow removal × Root	Vegetation × Root	Snow removal × Vegetation × Root
Nitrification ratio	$p < 0.05$	ns	ns	ns	ns	ns	ns
DOC/DON	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	ns	ns
MBC	ns	$p < 0.05$	ns	ns	ns	ns	$p < 0.05$
MBN	ns	$p < 0.05$	ns	ns	ns	ns	$p < 0.05$
Microbial C/N	ns	ns	ns	ns	ns	ns	ns

Table 4-4 Gross NH_4^+ production rate, ammonium consumption rate, gross NO_3^- production rate and nitrate consumption rate in the soil at the end of the incubation. Values represent means ± standard deviations of the mean (n = 6).

	Control				Snow removal			
	Oak soil		Larch soil		Oak soil		Larch soil	
Parameters (mg N kg^{-1} day^{-1})	Without Root	With Root	Without Root	With Root	Without Root	With Root	Without Root	With Root
Gross NH_4^+ production	1.55 ± 0.67	1.27 ± 0.67	1.31 ± 0.38	1.81 ± 0.37	2.10 ± 0.87	1.89 ± 0.67	1.55 ± 0.42	1.36 ± 0.47
NH_4^+ consumption rate	3.70 ± 3.30	2.49 ± 2.71	2.53 ± 1.69	1.98 ± 1.18	4.60 ± 3.58	2.49 ± 3.26	2.03 ± 1.00	1.76 ± 1.14
Gross NO_3^- production rate	0.74 ± 0.55	0.93 ± 0.43	0.80 ± 0.31	0.86 ± 0.21	0.47 ± 0.50	0.15 ± 0.08	0.71 ± 0.63	0.51 ± 0.37
NO_3^- consumption rate	0.00 ± 0.00	0.24 ± 0.33	1.01 ± 1.05	1.64 ± 1.00	0.24 ± 0.44	0.25 ± 0.43	0.47 ± 0.83	0.65 ± 1.01

Table 4-5 Results of ANOVA for gross NH_4^+ production rate, ammonium consumption rate, gross NO_3^- production rate, NO_3^- consumption rate. ns denotes not significant ($p > 0.05$).

Parameters	Snow removal	Vegetation	Root	Snow removal × Vegetation	Snow removal × Root	Vegetation × Root	Snow removal × Vegetation × Root
Gross NH_4^+ production rate	ns	ns	ns	ns	ns	ns	ns
NH_4^+ consumption rate	ns	ns	$p < 0.05$	ns	ns	ns	ns
Gross NO_3^- production rate	ns	ns	ns	ns	ns	ns	ns
NO_3^- consumption rate	ns	$p < 0.05$	ns	ns	ns	ns	ns

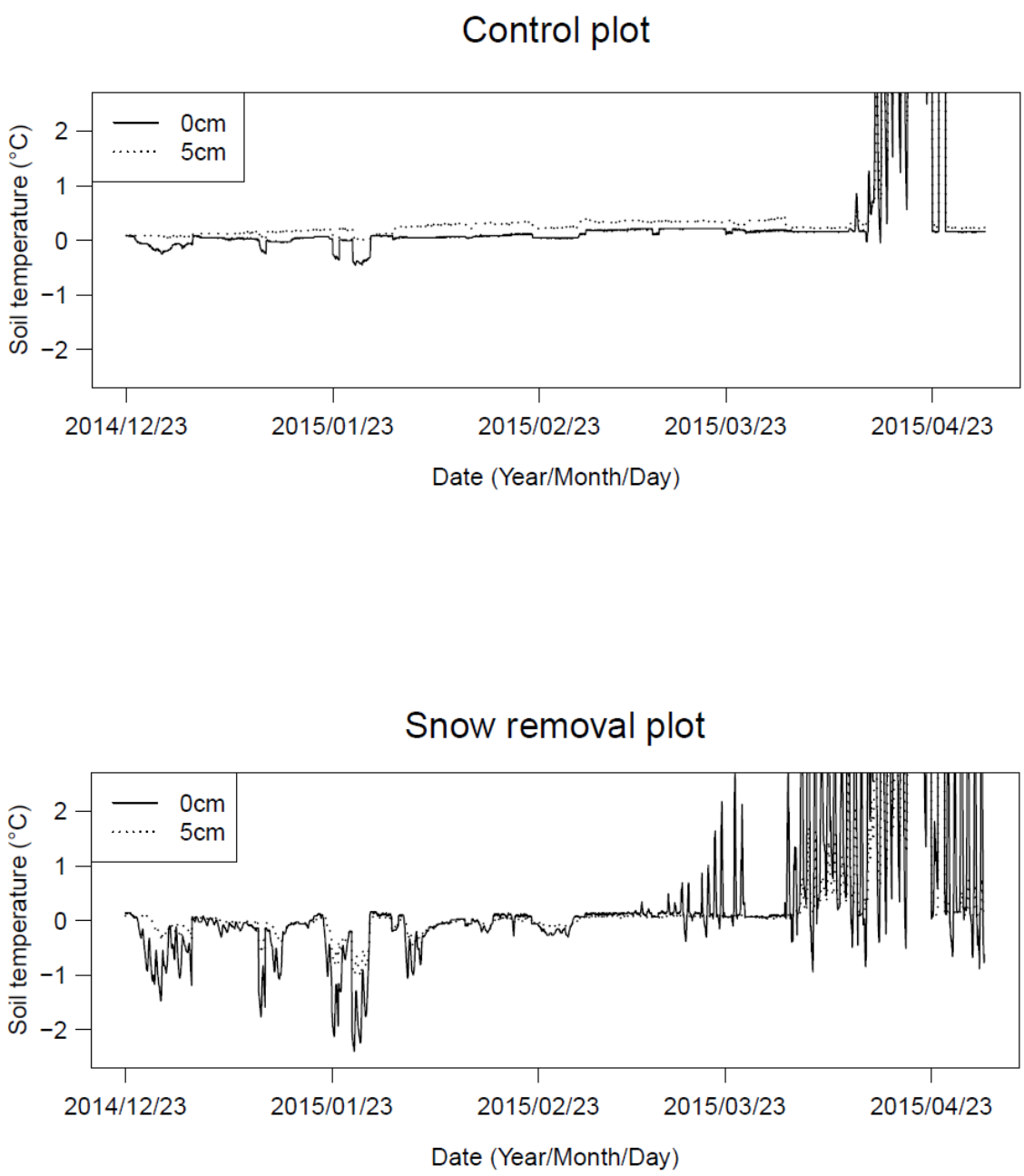


Fig. 4-1. Hourly mean soil temperature in control plot (upper) and snow removal plot (lower). Solid lines denote soil temperature at 0 cm depth from the soil surface. Dotted lines denote soil temperature at 5 cm depth from the soil surface.

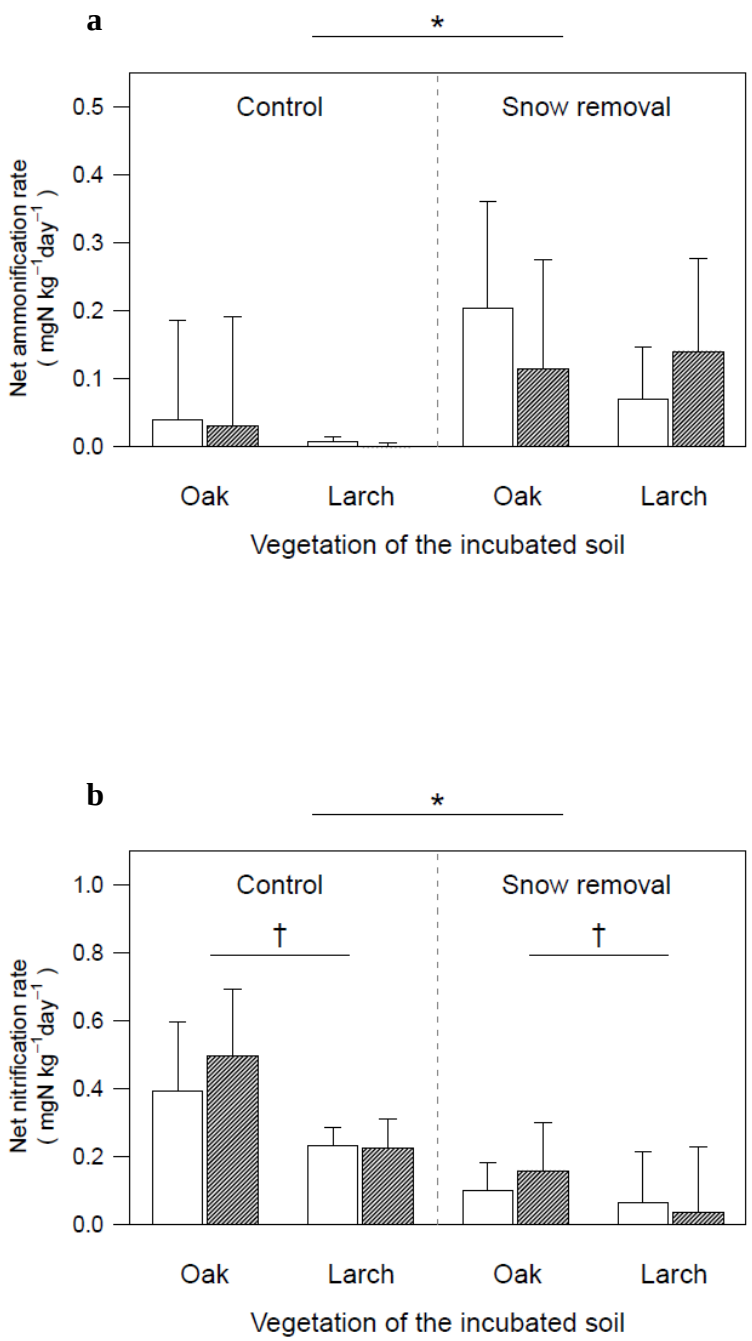


Fig. 4-2. Effects of snow removal and root addition on net NH_4^+ production rate (a) and net NO_3^- production rate (b). Values represent means and standard deviations of the mean (n = 6). White bars represent soil without root litter and filled bars represent soil with root litter. Asterisks and daggers denote significant differences with snow removal manipulation and vegetation type, respectively ($p < 0.05$).

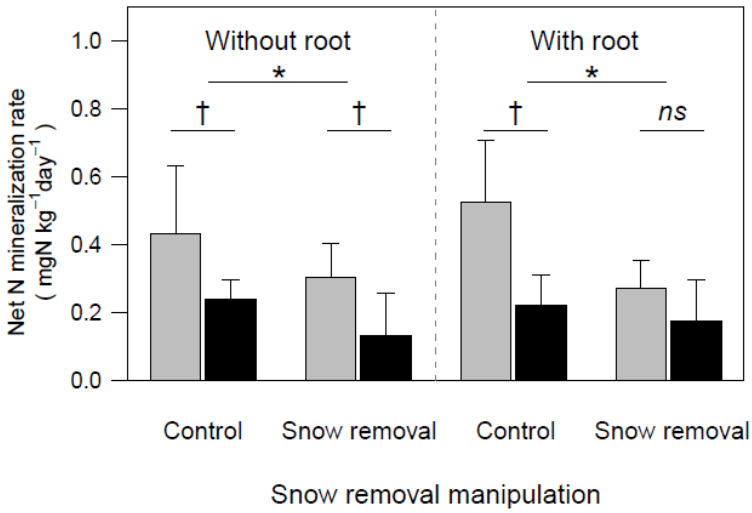


Fig. 4-3. Net N mineralization rate for the snow removal × vegetation type interaction in soil without/with root addition. Values represent means and standard deviations of the mean (n = 6). Gray bars represent oak soil and black bars represent larch soil. Asterisks and daggers denote significant differences with snow removal manipulation and vegetation type, respectively (p < 0.05). ns denotes not significant.

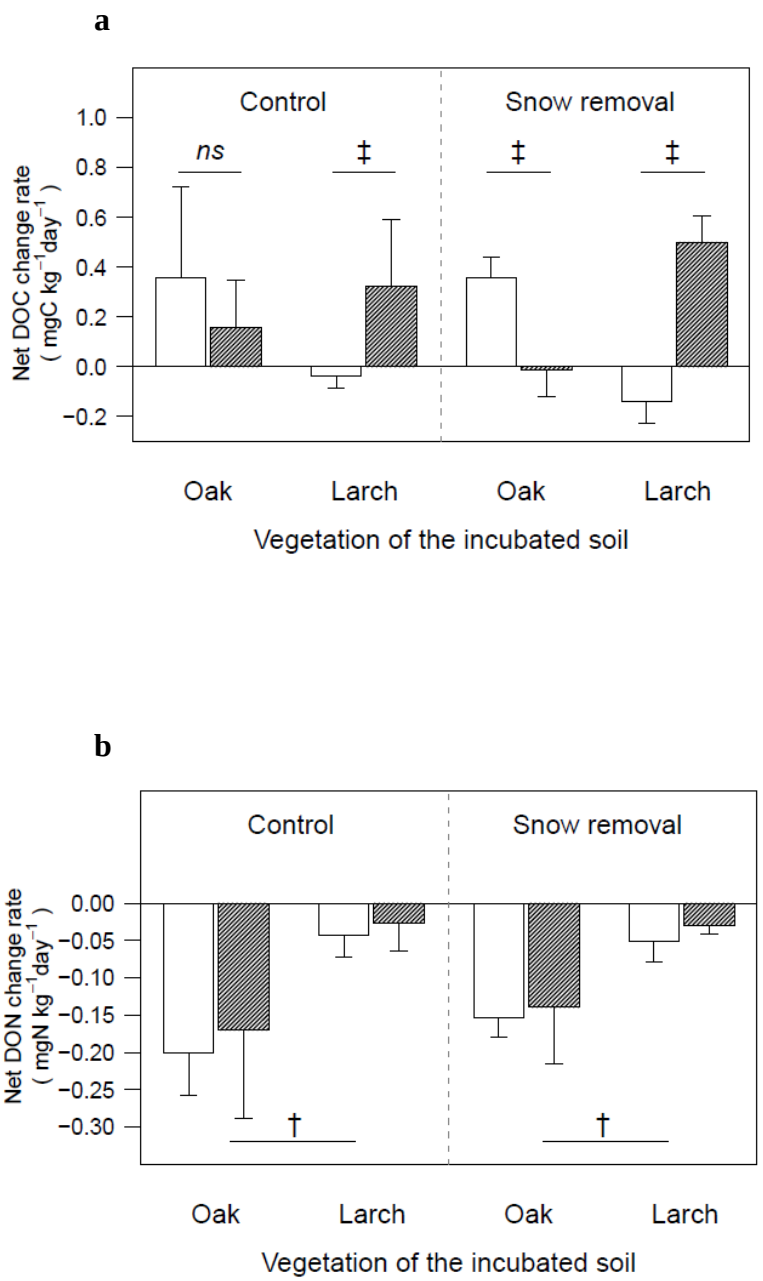


Fig. 4-4. Effects of snow removal and root addition on net rate of DOC change (a) and net rate of DON change (b). Values represent means and standard deviations of the mean (n = 6). White bars represent soil without root litter and filled bars represent soil with root litter. Daggers and double daggers denote significant differences between vegetation and root addition treatment, respectively ($p < 0.05$). ns denotes not significant.

Chapter 5
General discussion

5.1. Summary of the findings in the previous chapters

Winter climate change could alter N cycle in forest ecosystems. Soil freeze–thaw cycle altered by winter climate change is considered to affect soil nitrogen (N) transformations. Previous studies often reported that soil freeze–thaw reduce nitrification but increase ammonification (e.g., Shibata et al., 2013; Hishi et al., 2014). However, these response of soil N transformations and the reduction degree to soil freeze–thaw are varied among vegetation above the soil, and the variation is considered to relate with dissolved organic carbon (DOC) dynamics in the soil (Groffman et al., 2011, 2012). At the same time, plant root litter is a source of substrate and energy for soil microbes and considered to be increased by the soil freeze–thaw due to winter climate change. Plant species of root litter could affect the soil N transformations through its morphology and chemical component. Soil freeze–thaw could degrade litter and facilitate its decomposability (Hobbie and Chapin 1996). Thus, the relationship between root litter and soil N transformations perhaps change by soil freeze–thaw under winter climate change. In this thesis, I clarified the effect of soil freeze–thaw caused by winter climate change on soil N transformations focusing the role of root litter input as a mediator of effect of soil freeze–thaw. The overarching question was "How does soil freeze–thaw alter soil N transformations through input of root litter?" To accomplish the purpose, I addressed following specific questions.

Q1. Does the different plant species of fine root litter affect to soil N transformations under freeze–thaw cycles caused by snowpack decrease?

Q2. How does soil N transformations under different vegetation response to amplified soil freeze–thaw events caused by snowpack decrease with fine root litter input?

I conducted laboratory and field incubation studies with snow removal manipulation to address the above questions as describes in the Chapter 2–4, and I found the following points. First point was relating to the study site information (Chapter 2) that was necessary to develop the general discussion with other findings in the Chapter 3 and 4.

1. Snow removal manipulation did not cause significant changes in root biomass and necromass, total C and N content in root for both oak root and Sasa root in this site (Fig. 2-1 and Table 2-1), while the negative effect of soil freeze–thaw on root dynamics was reported elsewhere (Tierney et al., 2001; Gaul et al., 2008). Although there is possibility that the vegetation in my site (i.e., oak and Sasa) was tolerant to soil freeze–thaw, the variety of spatial and temporal among the sampling mask the difference.

2. Soil freeze–thaw events significantly increased net NH_4^+ production and net N mineralization (NH_4^+ +

NO_3^-) while it significantly reduced net NO_3^- production in soil in the entire incubation (1 week freeze–thaw followed by 1 week thawing). Although there was no significant difference of soil N transformation between oak root and Sasa root in the entire incubation period, the addition of both root litters caused significant differences in temporal change of soil N transformations (Fig. 3-1). Sasa root caused high and ephemeral increase during soil freezing periods whereas oak root litter caused moderate and continuous increase in net N transformations during following melting periods (Fig. 3-2). Different temporal change of promotion of gross N transformations by the species of root litter and microbial biomass compare to net N transformations suggest that contrasting interaction between microbial activity and nutrient from root litter was occurred in the two different plant species (Table 3-4). It was suggested that both of the two root species could cause surplus of net soil N transformations in subsequent the incubation (Chapter 3).

3. Soil freeze–thaw event enhanced by snow removal reduced net NO_3^- production and N mineralization but increased net NH_4^+ production in the oak forest and the larch forest. The magnitude of decrease in the net N mineralization were different among vegetation on soil with addition of Sasa root litter. The reduction in net N mineralization was smaller in soil under the larch forest than that under the oak forest (Fig. 4-3). In addition, Sasa root addition caused contrast changes on DOC dynamics during winter (Fig. 4-4). These contrast response between vegetation might be caused by difference of microbial activity (through decomposition and C use) and frost hardness of microbial community in the forest. Contrast response of DOC dynamics by vegetation suggest that soil freeze–thaw cause contrast change of soil N transformations in the longer time period (i.e., growing season and annual). Further, it was suggested that these contrast change by vegetation indicate the effect of soil freeze–thaw would be offset at the land scape scale. (Chapter 4).

Based on these findings, I would discuss the mechanism and the over winter effect of soil freeze–thaw on soil N transformations through root litter input.

5.2. Effect of plant species of root litter and forest above the soil on interaction between DOC and soil N transformations under soil freeze–thaw cycle

Some previous studies suggested that DOC could reduce net nitrification rate through increase in NO_3^- consumption and/or denitrification (e.g., Sobczak et al., 2003; Groffman et al., 2011; Shibata et al., 2013). However, in this study, regardless the plant species, the relationship between DOC supply from root litter and NO_3^- consumption rate in soil was not clear (Chapter 3, Figs. 5-1 and 5-2). Although the degree of the promotion of net N mineralization rate by Sasa root litter input with soil freeze–thaw was similar to that in oak root litter (Fig. 3-2), soil N transformations in the end of the incubation (soil freeze–thaw followed by thawing) was different between root litter species (Figs 5-1b and 5-2b). Similarly, the relationship between

DOC and nitrification was not clear in soils under the oak forest and the larch forest (Chapter 4). However, the effect of soil freeze–thaw and DOC transformations through root litter input on soil N transformations were distinct between the soils under the two forest. In the oak forest, root litter input caused net DOC consumption and the decrease of net N mineralization was larger than the larch forest in snow removal plot (Fig. 5-3a). Contrary to this, in the larch forest, root litter input caused net DOC production and decrease of net N mineralization was smaller than the oak forest in snow removal plot (Fig. 5-3b). Although I did not analyze soil N transformations of larch forest in the laboratory, the field incubation study in Chapter 4 imply the contrasting response of gross N transformations via DOC from root litter input in soils under the larch forest compare to the oak forest (Fig. 5-3). Similarly, contrasting DOC change to soil freeze–thaw and root litter input in soils under larch forest also imply the contrasting change of soil N transformations during the following growing season (Fig. 5-3b). These unclear relationship between DOC and NO_3^- transformations might because of soil microbes relate to nitrification is weak for low temperature (Cookson et al., 2002; Dalias et al., 2002). Fuss et al. (2016) implied that DOC regulation of nitrification might occur growing season rather than dormant season based on the stream study. Further, Haei et al. (2013) showed that DOC concentration in soil during growing season increased by soil freeze–thaw including increase in root mortality, and the enhanced DOC increased microbial respiration during growing season. Hence, the altered DOC pools by soil freeze–thaw and root litter input could affect soil N transformations during following growing season (Fig. 5-4).

5.3. Role of Sasa as an understory vegetation on N cycle in northern forest ecosystems through root litter input under winter climate change

In this thesis, I compared root litter of oak and Sasa which consist different biomass in the forest ecosystems. Although aboveground and belowground biomass of oak were larger than Sasa, root necromass of the two species were similar (Chapter 2). This result suggests that understory plant, Sasa significantly contribute to N cycling in this site as Fukuzawa et al. (2013) also suggested it in northern Hokkaido. The understory Sasa showed early increase of soil N mineralization immediately after soil freeze–thaw whereas overstory oak showed delayed increase of soil N mineralization after freeze–thaw (Chapter 3). These differences seems to because of different hardiness of root litter and its C and N content between the two plants. Thus, it was suggested that the understory plants contribute to instantaneous flush of soil N mineralization under winter climate change. Role of Sasa on N cycling in the forest ecosystem has been reported (e.g., high N uptake ability; Fukuzawa et al., 2006, low decomposition rate of Sasa leaf litter than tree leaf litter; Watanabe et al., 2013) and relative rapid N release from root than other plant part of *Sasa kurilensis* and *Betula* litter (Tripathi et al., 2006). The rapid soil N mineralization promotion by Sasa root litter input in this study consist with Tripathi et al. (2006), and implying understory Sasa might promote rapid N cycling in forest ecosystems. These findings also imply the significance of the understory plants on forest N cycling under future climate change.

5.4. Over-winter effect of soil freeze–thaw on N cycle in soils under the two vegetation through root litter input

Contrary to the soil N transformations during soil freeze–thaw period, my study suggested that root litter would affect soil N transformations during the subsequent periods.

In the laboratory experiment, the promotion effect of oak and Sasa root litter suggested root litter input possibly increase the subsequent soil N transformations in soil under oak forest. Net N mineralization and DOC production in soil under oak forest during winter decreased by soil freeze–thaw event with both oak and Sasa root litter (Figs 5-1 and 5-2). It was suggested that the reduction of net N mineralization and DOC production increased soil N transformations during the subsequent melting season. In addition, increase of the decomposition of root litter suggest that residual root litter might decrease in soil during the subsequent period. It was also suggested that recalcitrant root litter contribute to soil C and N budget for long time, resulting to diminish residual root that might affect C and N stocks in soil.

On the other hand, soil under larch forest might increase soil N transformations during the subsequent growing season by both larch and Sasa. Soil N transformations are generally influenced by the stoichiometric balance (i.e., C/N ratio). The C/N ratio of larch root (TC: 49.0 ± 1.84%, TN: 1.71 ± 0.39% and C/N: 29.8 ± 7.39, n = 3) was similar to that of Sasa root (TC: 44.7 ± 0.74%, TN: 1.33 ± 0.16% and C/N: 33.9 ± 3.70, n = 3). Thus, the effect of root addition on soil N transformations and DOC production might be similar. In this study, net DOC production in soil under larch increased during winter by soil freeze–thaw with Sasa root input (Fig. 5-3b). It was suggested that the increased DOC reduce net N mineralization and nitrification through enhance immobilization and denitrification (Groffman et al., 2011; Fuss et al., 2016).

If increase in root litter could increase N loss from the ecosystem during the spring time regardless of the species of plant root, altered DOC dynamics might cause different consequence in the growing season and annual. DOC dynamics was strongly affected by vegetation type, implying different effect of microbial composition among plant species. Thus, increase of root litter input by winter climate change might cause promotion of soil N mineralization in soil under larch forest (Fig. 5-3b). The various responses to winter climate change and root litter input suggest the probability that the influence of the effect of soil freeze–thaw on soil N dynamics would be offset between plant species.

At the same time, this study suggested that soil C and N stock could decrease by winter climate change. Spring time is considered to cause significant nutrient loss from forest ecosystems with runoff (Boyer et al., 2000; Sebestyen et al., 2009; Campbell et al., 2014). The increased N mineralization by soil freeze–thaw and root litter (Chapter 3 and 4) indicate increase in mobile inorganic N. In addition, the promotion of soil N transformations by root litter input indicate increase in decomposability of root litter by soil freeze–thaw (Chapter 3). It was suggested that the increase in residual root litter could contribute to decrease in soil C and N stocks. Mechanism of accumulation of soil organic matter (SOM) and its relation with litter decomposability are controversy among previous studies (e.g., Prescott 2010; Cotrufo et al., 2013; Hobbie et al., 2015). Microbial Efficiency-Matrix Stabilization frame work hypothesize that labile plant litter more contribute to SOM than recalcitrant litter, because microbial utilization was significant mechanism for

organic matter stabilization in soil (Cotrufo et al., 2013). However, Pries et al. (2017) reported that remaining C and N from *Pinus ponderosa* root litter in soil was larger than that of leaf litter in the foothills of the Sierra Nevada, western USA. Their 10 years litter decomposition experiment showed that the free light fraction which is not connected with soil minerals was large sink of the litter derived C and N. Further, although the ratio of root derived component in free light fraction was larger than that of leaf litter, the difference of the ratio was not different in finer particulate fraction. This result indicate that low decomposability litter is not necessarily accumulate in soil. Thus, consequence of increase in root litter decomposability could reduce soil C and N budget.

Durán et al. (2016) suggested that in situ NH_4^+ production and nitrification decreased with increasing soil freeze–thaw, and thus N availability in a northern hardwood forest would be lower under future climate change. The suggested perspective of soil N transformations under winter climate change in this study (Fig. 5-4) are mostly consistent with their previous findings. On the other hand, Sorensen et al. (2016a and b) implied that soil freeze–thaw caused by winter climate change perhaps reduce soil C and N stocks through inhibiting soil enzyme production and activity, although the effect on soil enzyme was limited to spring time (Sorensen et al., 2016a). I did not analyze soil enzyme activity, but my contrast result of the soil N transformations by vegetation claim that the possible reduction of soil C and N stocks because of diminished root litter residue by soil freeze–thaw under winter climate change traced different reduction processes between the vegetation. The reduction of the N mineralization rate in the consequence for the long-time period should include various mechanisms. The variety response of C response to the winter climate change and root litter input by vegetation in this study suggest that function of the soil microbial community is also considerable.

5.5. Research limitations and future research needs

Finally, I would note the limitation in this study. Important factor for N cycling in forest ecosystems and soil C and N stocks are i) feedback from plant including compensate growth in the growing season (Reinmann et al., 2016; Sorensen et al. 2016b), ii) effect of Andosol on nutrient dynamics (Huygens et al., 2011; Miyazawa et al., 2013; Urakawa et al., 2014) and iii) effect of silicate in Sasa litter on cycling and accumulation of C and N in soil (Wagai et al. 2013; Watanabe et al., 2013; Song et al., 2017).

Sorensen et al. (2016b) showed significant root compensate regrowth was caused by soil freeze–thaw in the growing season. On the other hand, Reinmann and Templer (2016) showed that soil freeze–thaw reduced root biomass but increased basal area growth of *Acer rubrum*. Other study reported that soil freeze–thaw reduced shoot growth and increased foliar starch concentrations of *Acer saccharum* (Comerford et al., 2013). These changes of plant performance could affect processes in soil (Kreyling 2010), and the interaction between plant and soil might causes the effect of climate change diverse (Groffman et al., 2012; Makoto et al., 2013).

Volcanic soil such as Andosol strongly adsorb organic matter (Miyazawa et al., 2013; Urakawa et al.,

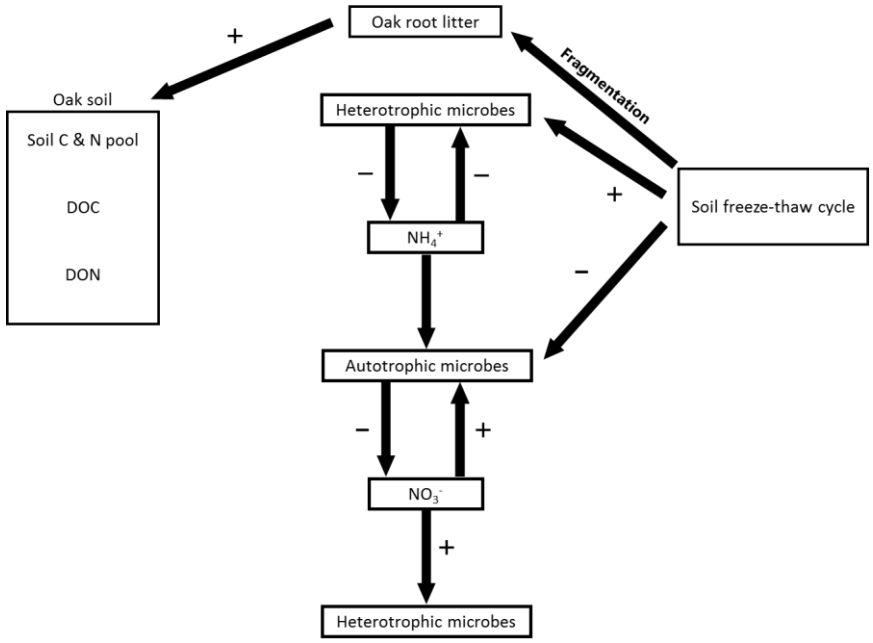
2014). High SOM accumulation in volcanic soil cause high soil N mineralization rate in Japan (Urakawa et al., 2014). However, Huygens et al. (2011) reported that organic N were partly adsorbed with soil minerals and the organic matter were relatively resistance for N mineralization in an Andosols in Chile. The relationship among sorption of N and organic matter in soil and N cycling in forest ecosystems is controversy, but the effect might present. Thus, the testing of the effect of the root litter input on soil N transformations under soil freeze–thaw in other soil type is necessary.

Sasa dwarf bamboo is a significant component in forest ecosystems at Hokkaido. Sasa is a poaceous plant that is highly accumulate silicate in their plant body (Watanabe et al., 2013). Recent studies have argued that silicate and phytolish are significant adsorption for organic matter which could affect nutrient cycling and C and N accumulation in soil (Wagai et al., 2013; Song et al., 2017). Thus, increase in decomposability of Sasa root by intensified soil freeze–thaw could affect N cycling and nutrient accumulation in this area. Further research is needed to clarify the effect of silicate in Sasa on N cycling in forest ecosystems under climate change.

5.6. Conclusion

Winter climate change is predicted to decrease snow accumulation resulting to alter soil freeze–thaw regime. However, the effect of soil freeze–thaw on soil N transformations has not been fully understood yet. In this thesis, I focused on the role of root litter input on soil N transformations under winter climate change. The effect of soil freeze–thaw on soil N transformations through root litter was equivalent in quantitatively by plant species of root litter, whereas soil N transformation rates showed distinct temporal changes by plant species. Labile Sasa root litter caused higher and ephemeral promotion of soil N mineralization rate, while recalcitrant oak root litter caused moderate and durable promotion. At the same time, the response of soil N transformations to soil freeze–thaw and root litter input was different by vegetation (oak forest vs. larch forest) on the soil. It is suggested that the difference by the vegetation caused by DOC change in each vegetation soil. Contrast responses of soil DOC and N transformations by the vegetation possibly cause the balanced N cycling in the landscape level in growing season. It is predicted that increase in root litter input might affect soil N transformations annually regardless the two plant species, while the alternation of the soil N transformations might be different by soil vegetation. It is suggested that root litter play a role under winter climate change as a driver of altering soil N transformations in quantity and temporally and of changing specific soil DOC and N transformations in the vegetation.

a) Freeze–thaw period



b) Thawing period

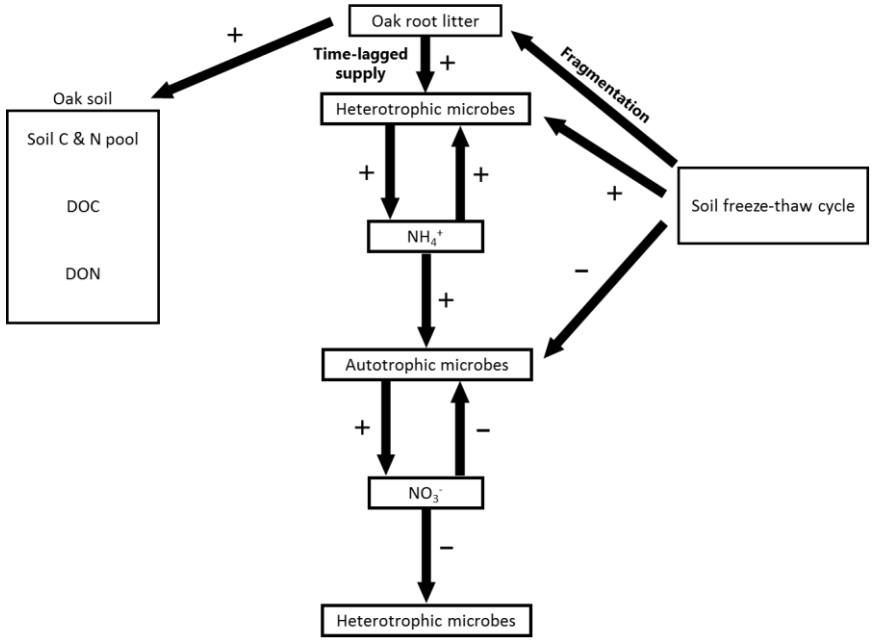
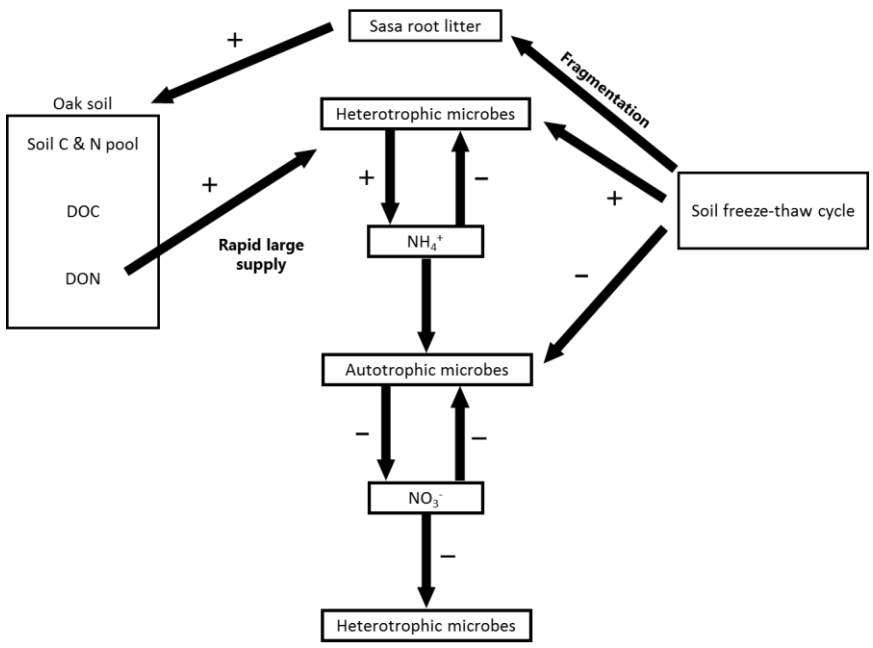


Fig. 5-1. Summary of the findings in Chapter 3. Effect of the soil freeze–thaw and oak root litter on nitrogen transformations during a) freeze–thaw period and b) thawing period in soils under the oak forest. Plus signs and minus signs indicate positive (promotion) effect and negative (inhibition) effect, respectively.

a) Freeze–thaw period



b) Thawing period

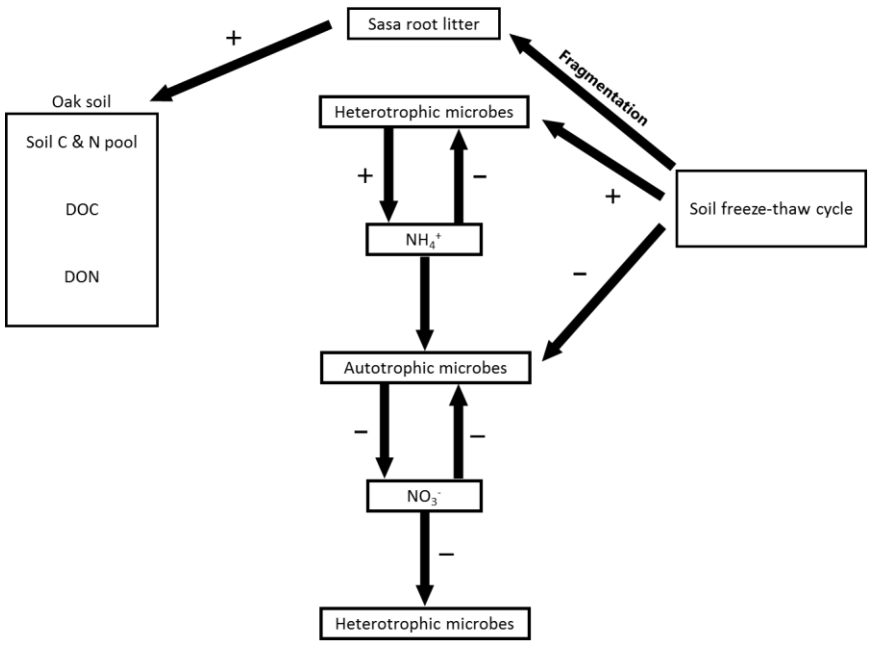
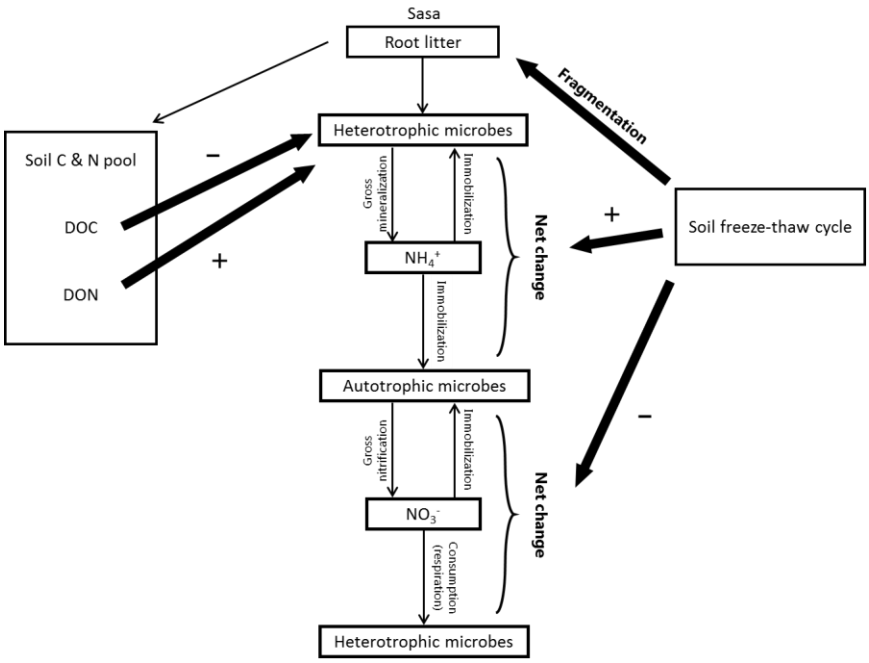


Fig. 5-2. Summary of the findings in Chapter 3. Effect of the soil freeze–thaw and Sasa root litter on nitrogen transformations during a) freeze–thaw period and b) thawing period in soils under the oak forest. Plus signs and minus signs indicate positive (promotion) effect and negative (inhibition) effect, respectively.

a) Oak forest soil with Sasa root litter



b) Larch forest soil with Sasa root litter

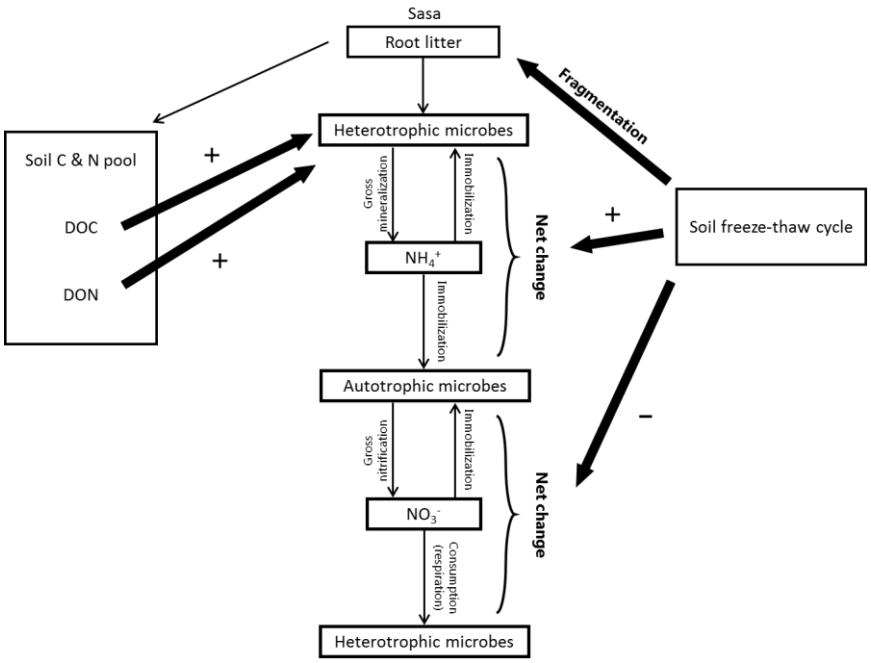
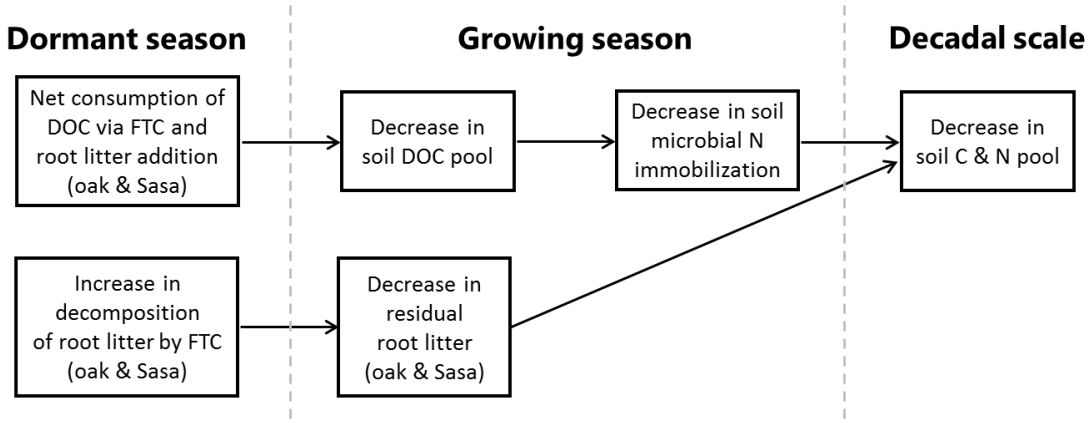


Fig. 5-3. Summary of the findings in Chapter 4. Effect of the soil freeze–thaw and Sasa root litter on nitrogen transformations in soils under a) the oak forest and b) the larch forest during the dormant season. Plus signs and minus signs indicate positive (promotion) effect and negative (inhibition) effect, respectively.

a) Oak forest



b) Larch forest

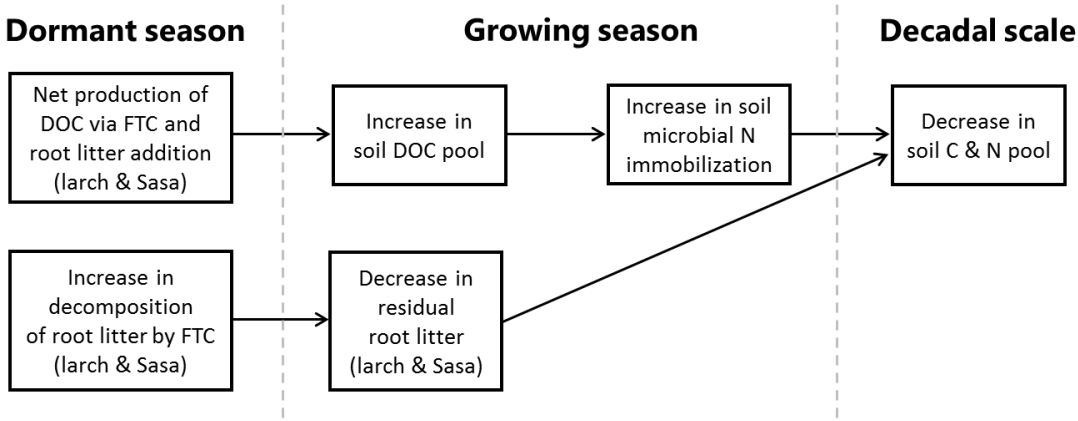


Fig. 5-4. Prediction of the effect of the soil freeze–thaw and root litter on soil nitrogen transformations during dormant season on soil C and N cycle following growing season and decadal scale under a) the oak forest and b) the larch forest. FTC: soil freeze–thaw cycle altered by winter climate change.

Supplementary material

Preparatory study for laboratory incubation in Chapter 3

S1.1. Introduction

The effect of soil freeze–thaw on C and N dynamics has been received much attentions by world researchers across the world. Soil freeze–thaw is fluctuation of temperature, thus the effect is expected to be highly varied by the pattern (e.g., duration, frequency of freeze–thaw and temperature range).

Condition of laboratory incubation highly affect the result as mentioned above (Henry 2007; Matzner and Borken 2008). For example, Haei et al. (2012) showed that DOC decomposability was affected by frost duration in the field, whereas that was strongly affected by frozen intensity in the laboratory incubation. Austnes and Vestgarden (2008) compared C and N leachate from soil under some freeze–thaw regimes with constant frozen and not frozen soil. Against their expectations, C and N leachate increased significantly in soil under constant frozen, and changes under freeze–thaw regimes were not significant. Further, Klaminder et al. (2013) showed relationship between humus mixture and soil respiration was changed by increase in incubation temperature and the result imply the effect of freeze–thaw on following thawing regime is highly dependent on the thawing period.

Although there are various manifest and latent variables affect soil N transformations under freeze–thaw condition, given the prediction of winter climate change by modeling (Inoue and Yokoyama 1998) and reviewing (Park et al., 2010; Shibata 2016) suggest necessity of narrowing and focusing of parameters to verify. In line with this thinking, I explore the parameters to I should focus on the laboratory experiment according to the previous study conducted in my study site (Christopher et al., 2008).

In this supplementary, I conducted laboratory incubation in some freeze–thaw regime that are simulating current temperature regime which also can be a dormant regime in the future climate change, to explore the influential temperature regime in my study site. Further, I compared two temperature range and thawing period following the freeze–thaw to explore adequate incubation temperature and duration of posttreatment.

S2. Material and methods

S2.1. Sampling of soil and root

The soil collection and processing were described in Chapter 3.

S2.2. Temperature setting

Soils in Shibecha experimental forest experience seasonal soil frost yearly. I extracted soil freeze–thaw pattern from soil temperature showed in Christopher et al. (2008). In early December 2004, surface soil

experienced 5 times soil freeze–thaw ranged from −8 °C to 0 °C in approx. 12 h interval (day time and night). 5 cm depth soil experienced less intensity of the freeze–thaw, ranged from approx. −3 °C to 0 °C in the same time. During December to April when is the normal snow season in this area, 5 cm depth soil in upper slope experienced 2 times soil freeze–thaw ranged from approx. −2 °C to 1 °C. In the entire snow time, surface soil in upper and lower slope experienced one large freeze–thaw in minimum temperature −5 °C. Thus, I extracted 2 types of freeze–thaw range; lower than 0 °C and over 0 °C. Further, the over 0 °C freeze–thaw can be separated into two temperature regimes; frequent and once. To combine these pattern, I narrowed 3 types of freeze–thaw regime (1) frequent freeze–thaw with maximum temperature is 0 °C, (2) frequent freeze–thaw with maximum temperature is over 0 °C and (3) once freeze–thaw with maximum temperature is over 0 °C.

S2.3. Laboratory incubation and measuring potential leachate of nutrient from root litter

The soil incubation experiment was carried out in a 100 ml glass bottle. I placed approximate 25 g of soil into the glass with four replication for each manipulation.

I conducted two incubation experiment. In the first experiment, I added 15 mg g soil^{-1} of root into the bottle and stirred well. The soil were exposed to four different temperature regimes: +5 °C to −5 °C (extreme freeze–thaw, Extreme FT), −5 °C to 0 °C (moderate freeze–thaw, Moderate FT), −5 °C constant (Frost). As a control treatment, +5 °C constant (Non frost) for 7 days with soil without root was conducted. After these treatments, all soils were incubated at +5 °C for 2 days.

In the second experiment, I prepared 3 grade of root addition treatment; 15 mg g soil^{-1}, 5 mg g soil^{-1} and 0 mg g soil^{-1}. The soils were exposed to two temperature regimes: +5 °C to −5 °C (extreme freeze–thaw, Extreme FT) and −5 °C constant (Frost) for 7 days followed by 7 day thawing. Soils were exposed to 5 °C and 10 °C during thawing period, respectively.

To identify the effect of freeze–thaw on microbes and root litter fragmentation, I measured potential leaching from root litter in each temperature regime. Approximately 150 mg root litter of oak and Sasa were packed into two types of the glass bottle, respectively (n = 3). The root leachate was expected to be affected by water environment (i.e., water contents in a root litter and soil water surrounding the root), I estimated the potential root leachate as the mean of that in water filled environment and that of air filled environment. Thus, one bottle was added 50 ml of ultra-pure water (UPW), and the other was not added anything. The end of the freeze–thaw, incubated roots with water was placed in room temperature about 12 h until the ice was totally thawed. Then, the bottle was shaken for 1 h gently and the solution was percolated through a glass fiber filter (GF/F, Whatman Int. Ltd., Maidstone, Kent, UK). The roots without water was added 50 ml of UPW when the incubation ended and was shaken and percolated the same manner of water-added sample.

S2.4. Chemical analysis

The details were described in Chapter 3.

S2.5. Statistical analysis

I used multivariate analysis of variance (MANOVA) to assess the effect of temperature regime and difference by root species on potential nutrient leaching from root litter. The effect of temperature regime and root addition on net N transformations and DOC transformations were analyzed by two-way ANOVA. If the effect of temperature regime is significant, I conducted multiple comparison by Tukey's HSD. Further, if the interaction between the temperature regimes and root addition was significant, I applied multiple comparison among temperature regimes by root treatment (Tukey's HSD). The relationship between soil N transformations and root addition amount were analyzed by regression analysis by temperature regime and thawing temperature. All statistical analysis were conducted using R software (R Development Core Team, 2015). I used R package "car" for the ANOVA and "multcomp" for the multiple comparison, respectively.

S3. Results

S3.1. Potential nutrient leaching from root

Potential nutrient leaching from root liter was significantly different by root species (MANOVA, Table S1 and Fig. S1a, b), although the difference by temperature regimes was not significant. Leaching of DOC and DON were significant higher from Sasa than oak (Fig. S1a, b). These contrast DOC and DON caused significant higher DOC/DON from oak than Sasa (Fig. S1c). Potential leaching of inorganic N from Sasa was larger than oak (Table S1). The difference was caused by large leaching of NO_3^-. Contrary to NO_3^-, NH_4^+ from oak was higher than Sasa.

S3.2. Effect of temperature regime and root addition on soil N transformations and DOC transformation in the short time period

Root litter addition significantly increased net NH_4^+ production incubated at +5 °C for 2 days after all freeze–thaw treatments (15 mg added > 0mg added). NH_4^+ production rate was net consumed in Non frost treatment, and temperature regimes transfer the rate from consumption to production (Fig. S2a).The difference by temperature regimes was significant with maximum at Moderate FT treatment followed by Frost treatment. Similarly, net NO_3^- production rate was increased by root addition in each freeze–thaw regime (Fig. S2b). Net NO_3^- production rate was maximum at Extreme FT treatment and high in the order of Moderate FT and Frost treatment. Consequently, net N mineralization rate was increased by root addition in each treatment (Fig. S2c). Net N mineralization rate was maximum in Moderate FT followed by similar amount of Frost and Extreme FT, and Non frost. DON change rate was net consumed in all temperature regimes in soil without

root (Fig. S3 upper). Temperature regime decreased DOC consumption rate significantly. Root addition increased DOC change significantly except for Moderate FT treatment. This interaction caused higher DOC production in Extreme FT with root than other treatments. DON change rate was net consumed in all treatment (Fig. S3 lower). Net DON consumption was decreased by each temperature regimes than Non frost treatment. Root addition decreased consumption rate in all temperature regimes.

S3.3. The effect of thawing temperature on response of soil N transformations to root addition

The relationship between net NH_4^+ production rate during incubation initial to 2day thawing and root addition was significantly positive in Frost treatment thawing at 5 °C and 10 °C respectively (Fig. S4a). However, the relationship between net NH_4^+ production rate during thawing and root addition was significantly negative in Frost with 5 °C thawing and Extreme FT with thawing at 5 °C and 10 °C respectively (Fig. S4c). The relationship between net NH_4^+ production rate from incubation initial to 7day thawing and root addition was not significant in all treatments (Fig. S4b). Contrary to NH_4^+ production, relationship between net NO_3^- production rate and root addition was not significant except for Extreme FT thawing at 10 °C in net NO_3^- production during thawing period (Fig. S5). Relationship between net N mineralization from incubation initial to 2day thawing and root addition was positive in Frost 5 °C ($p < 0.05$) and 10 °C ($p = 0.06$) and Extreme FT at 10 °C ($p = 0.054$) respectively (Fig. S6a). Relationship between net N mineralization rate from incubation initial to 7day thawing and root addition was positive in Frost with 10 °C thawing (Fig. S6b), although there were no relationship in that of net NH_4^+ production rate and net NO_3^- production rate. Relationship between net N mineralization in thawing period and root addition was negative in Frost with 5°C thawing and Extreme FT with 10 °C thawing (Fig. S6c).

S4. Discussion

S4.1. Effect of temperature regime and root addition on soil N transformations and DOC transformation in the short time period

In this experiment, I observed highest net NH_4^+ production and net N mineralization in the temperature regime under 0 °C (moderate FT, Fig. S6). This result contrasting with laboratory incubation conducted similar temperature regime (Schimel and Clein 1996). Schimel and Clein (1996) showed that burst of microbial respiration was decreased with number of freeze–thaw in soils from tundra. Soil microbial community in the environment like Tundra where is severe winter condition is resistant to soil freeze–thaw than that in temperate ecosystems (Stres et al., 2010). My site is temperate forest so that the microbial resistance or physiology was different from Schimel and Clein (1996). Further, Tilston et al. (2010) showed that soil respiration declined rapidly at the end point of −2 °C imply the nearly 0 °C sub-zero temperature

remain high microbial activity. Thus, it is possible that moderate FT regime was adequate for the microbial activity in this soil.

S4.2. Effect of temperature and duration of post treatment thawing on the response of soil N transformation to root addition

Net NH_4^+ production was more affected by thawing temperature and duration than those in net NO_3^- production (Figs. S4 and S5). The higher response of ammonification to temperature increase is consistent with previous studies that clarify the temperature dependency (Q_{10}) of soil N transformations in subalpine forest ecosystems (Grenon et al., 2004) and in the temperate forest ecosystems (Schütt et al., 2014). The relationship between net NH_4^+ production rate and root addition was considerable affected by thawing temperature. Higher temperature (10 °C) might cause faster N mineralization rate because microbial growth and activity increase with temperature increasing ranged from 0 to 30 °C (Pietikäinen et al., 2005). Thus, the difference by freeze–thaw regime was varied by thawing temperature. Further, duration of thawing affect significantly net NH_4^+ production rate. The longer thawing period did not show the difference by freeze–thaw regime (Fig. S4b), although the short thawing time period did. This was because the contradicting temporal trends of the relation between temperature and soil N transformations was occurred during thawing period (Fig. S6c). Net N mineralization rate showed similar trends with net NH_4^+ production rate. Although the clear effect of freeze–thaw regime on net NH_4^+ production was observed, potential nutrient leaching from root was not different by freeze–thaw regime (Fig. S4, Table S1). This indicate that not the degree of the root fragmentation, but microbial activity affect soil N transformations.

Table S1 Potential leaching NH_4^+ and NO_3^- from root litter. Values represent means ± the standard deviations of the mean (n = 6). Asterisks denote significant differences by species of root litter (tested by MANOVA, $p < 0.05$).

	NH_4^+ (mg N g^{-1}) *		NO_3^- (mg N g^{-1}) *	
Temperature regime	Oak	Sasa	Oak	Sasa
Non frost	0.22 ± 0.09	0.13 ± 0.06	0.005 ± 0.004	0.60 ± 0.12
Frost	0.17 ± 0.06	0.10 ± 0.03	0.005 ± 0.003	0.64 ± 0.09
Moderate FT	0.18 ± 0.04	0.10 ± 0.04	0.004 ± 0.001	0.63 ± 0.09
Extreme FT	0.22 ± 0.06	0.11 ± 0.05	0.005 ± 0.002	0.66 ± 0.06

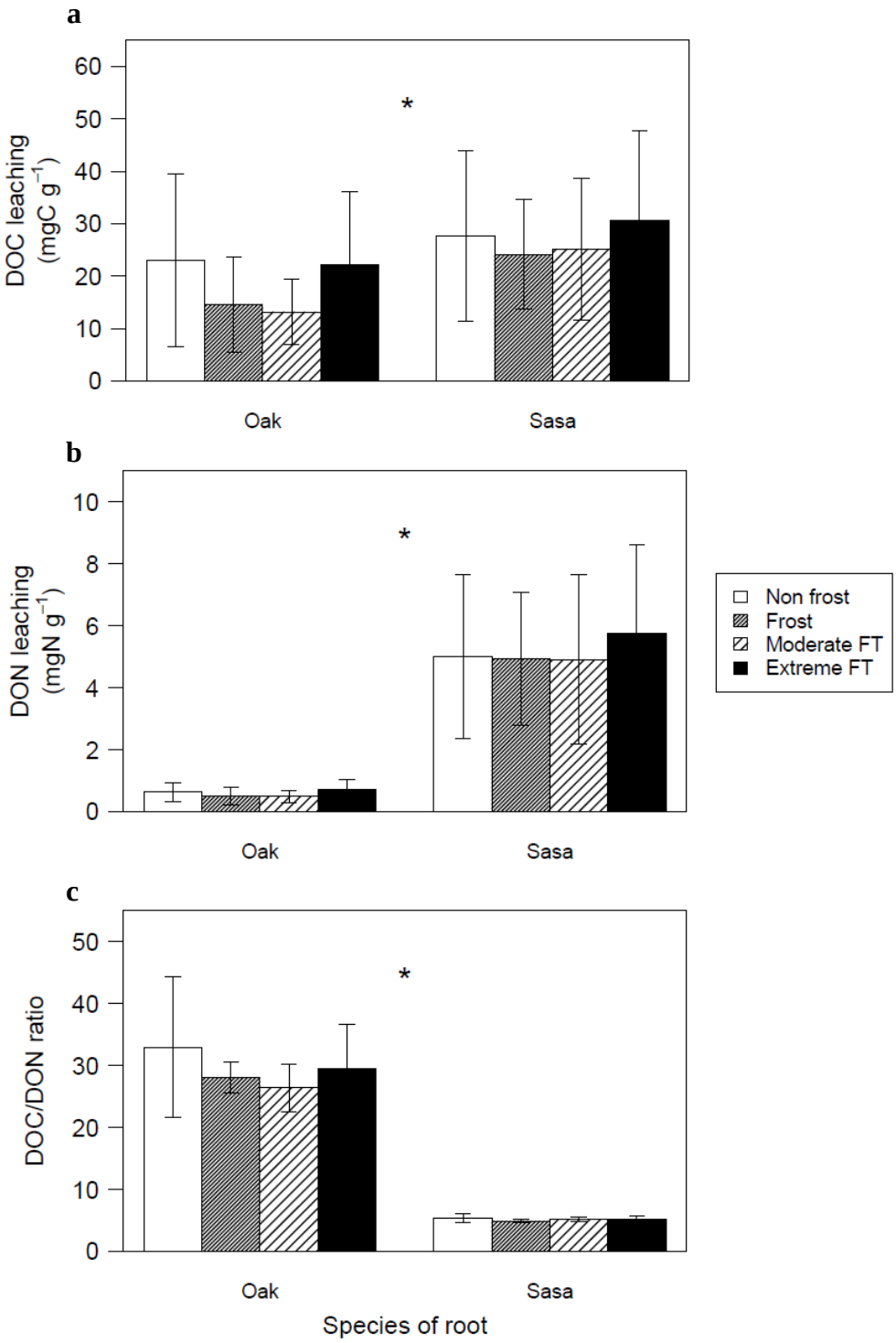


Fig. S1. Potential leaching of a) dissolved organic matter (DOC) and b) dissolved organic nitrogen (DON) from root litter by temperature regimes and c) DOC/DON ratio. Values represent means and the standard deviations of the mean (n = 6). Asterisks denote significant differences by root species ($p < 0.05$).

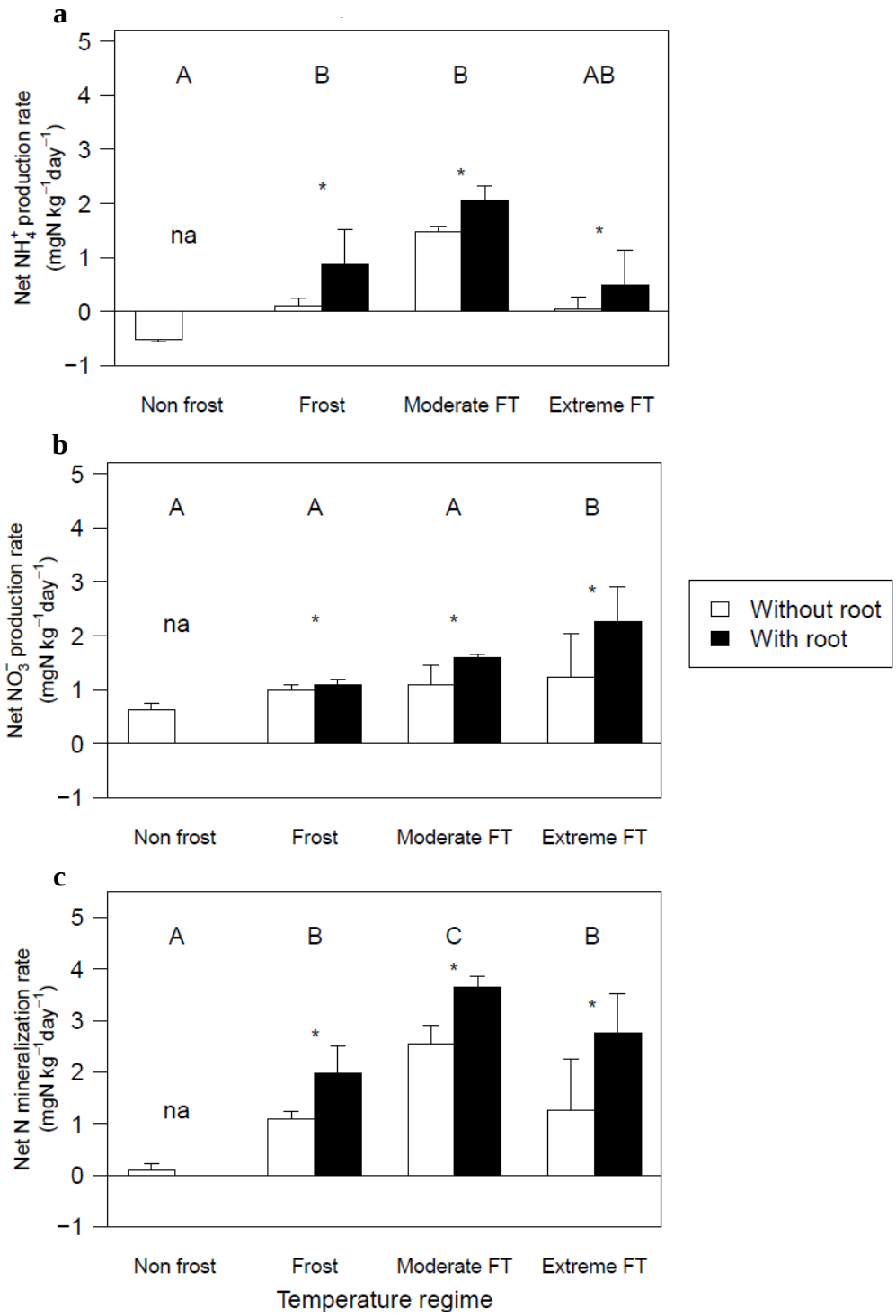


Fig. S2. Effect of temperature regimes on a) net NH_4^+ production rate, b) net NO_3^- production rate and c) net N mineralization rate in 2 day after thawing at 5 °C. Values represent means and the standard deviations of the mean (n = 4). White bars represent soil without root litter and filled bars represent soil with root litter. Asterisks denote significant differences with root addition (p < 0.05). Different upper cases indicate significant difference among temperature regimes (tested by Tukey's HSD, p < 0.05). na denotes not available.

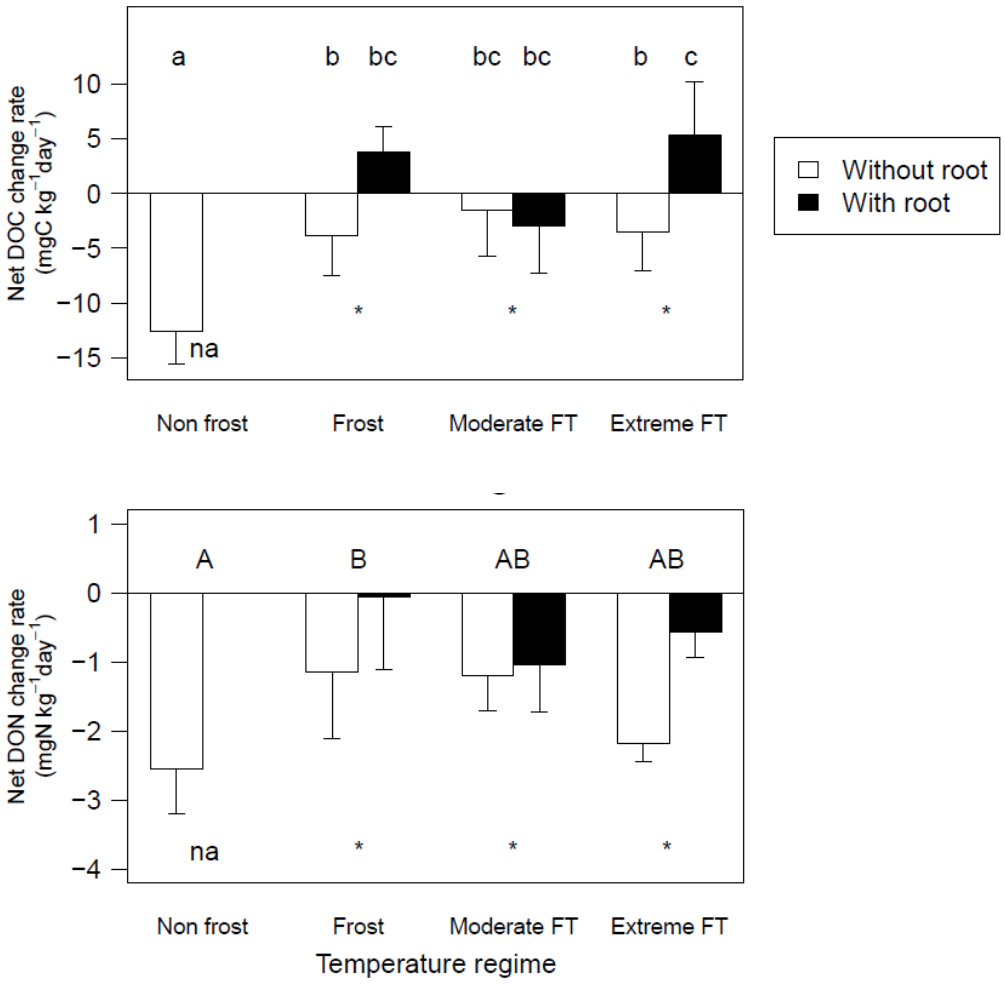


Fig. S3. Effect of temperature regimes on net DOC production rate (upper) and net DON production rate (lower) in 2 day after thawing at 5 °C. Values represent means and the standard deviations of the mean (n = 4). White bars represent soil without root litter and filled bars represent soil with root litter. Asterisks denote significant differences with root addition (p < 0.05). Different upper cases and lower cased indicate significant difference among temperature regimes and among temperature regimes and root treatment (tested by Tukey's HSD p < 0.05), respectively. na denotes not available.

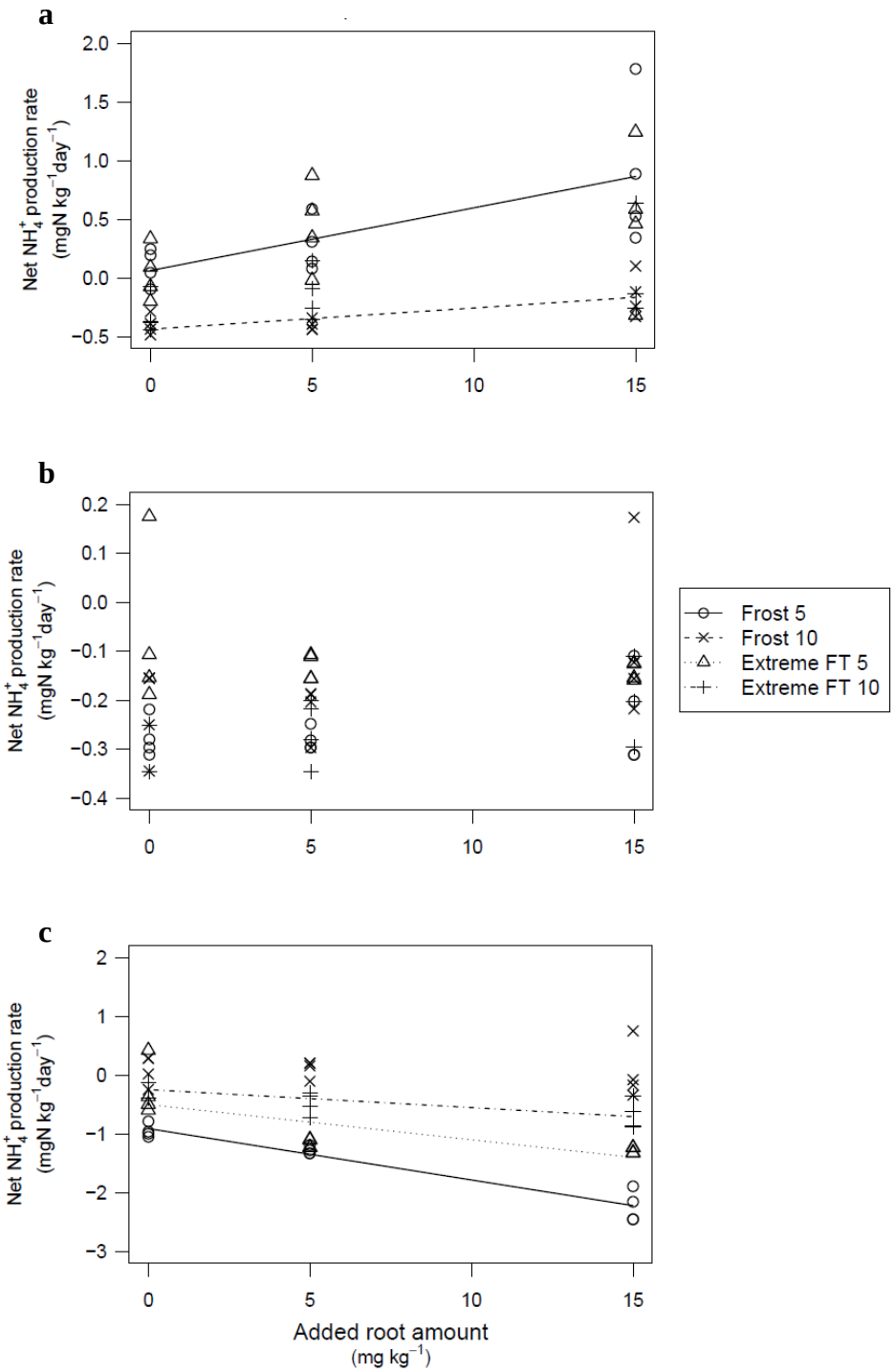


Fig. S4. Relationship between net NH_4^+ production rate and amount of root addition during a) from incubation initial to 2 day thawing, b) from incubation initial to 7 day thawing and c) thawing period under frost and extreme freeze–thaw (Extreme FT) regime and thawing at 5 °C and 10 °C. Lines indicate significant relationship between the N transformation rate and amount of root litter input in each treatment (n = 4).

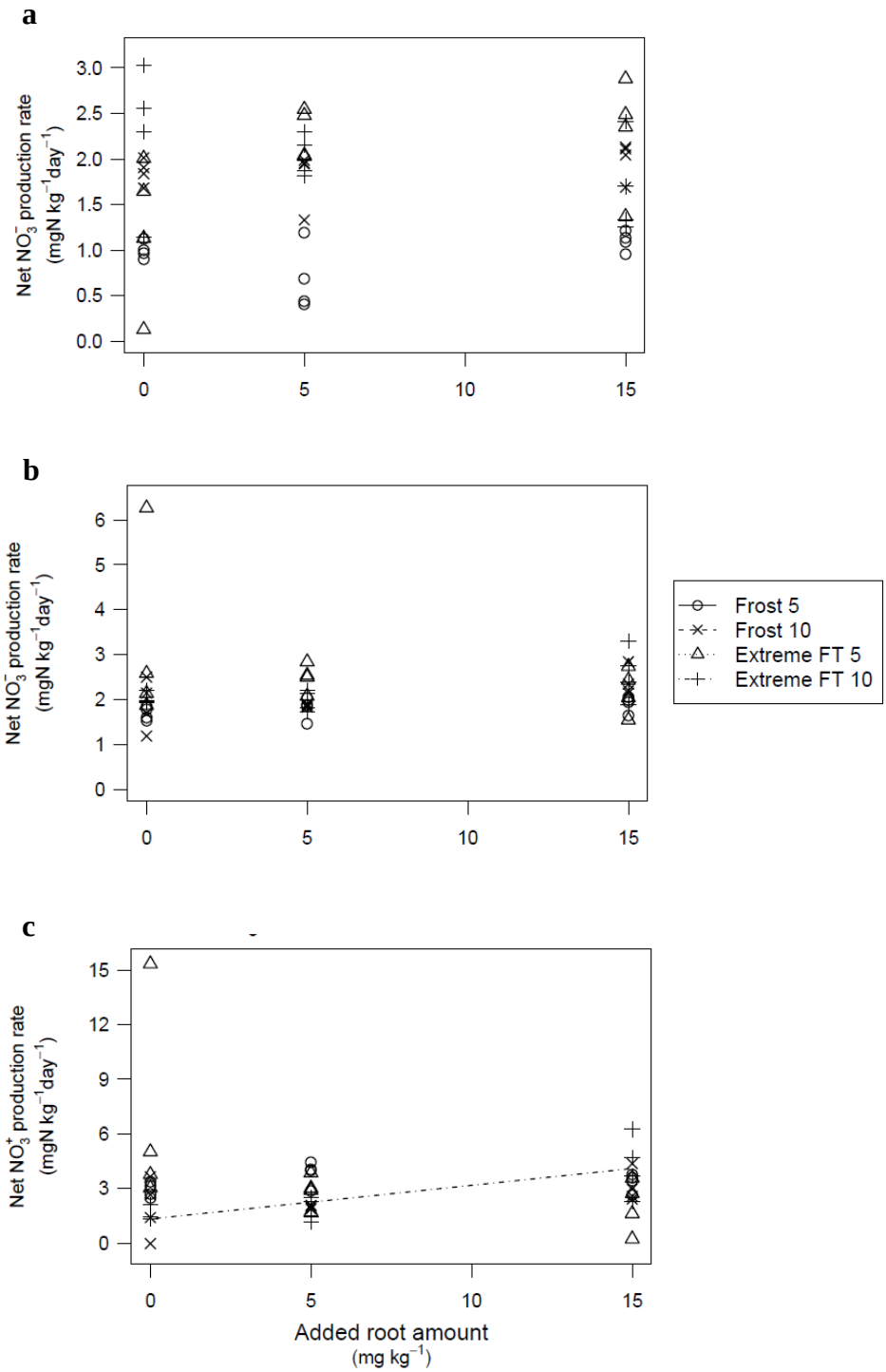


Fig. S5. Relationship between net NO_3^- production rate and amount of root addition during a) from incubation initial to 2 day thawing, b) from incubation initial to 7 day thawing and c) thawing period under frost and extreme freeze–thaw (Extreme FT) regime and thawing at 5 °C and 10 °C. Lines indicate significant relationship between the N transformation rate and amount of root litter input in each treatment (n = 4).

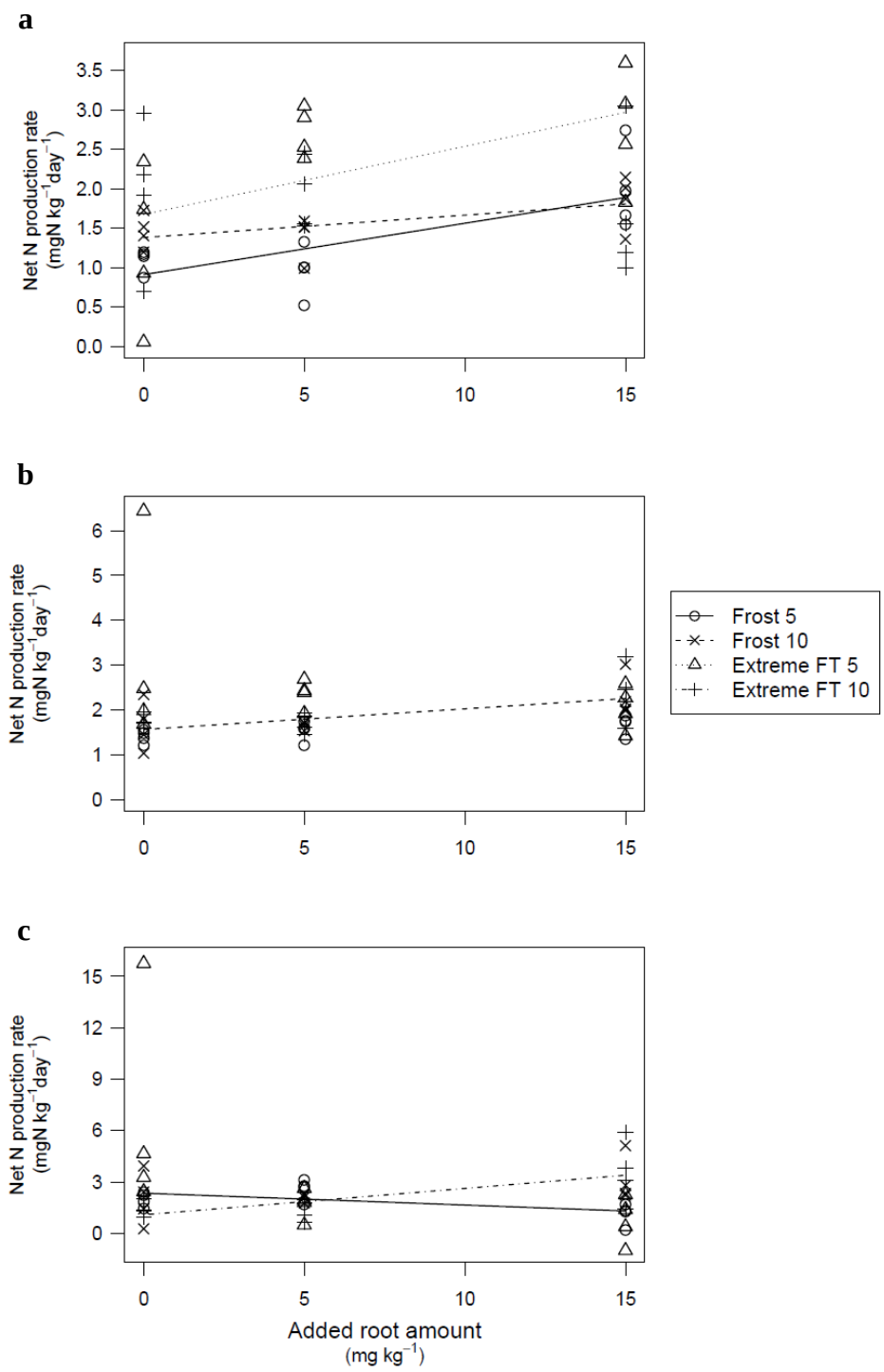


Fig. S6. Relationship between net N production rate and amount of root addition during a) from incubation initial to 2 day thawing, b) from incubation initial to 7 day thawing and c) thawing period under frost and extreme freeze–thaw (Extreme FT) regime and thawing at 5 °C and 10 °C. Lines indicate significant relationship between the N transformation rate and amount of root litter input in each treatment (n = 4).

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要旨

冬季の気候変動は、降雪の量やパターンの変化を通じて森林土壌の凍結融解レジームを変化させる。凍結融解の振幅域や頻度の増加は、植物細根や土壌微生物の損傷や死亡率の増加をもたらす。これらの変化は、冬季から春季にかけての森林生態系からの窒素流出の増加を引き起こすと考えられている。土壌の窒素変換速度に影響する要因を明らかにするためには、土壌微生物の活性や機能とともに、基質の量や利用性を考える必要がある。土壌や有機物に含まれる窒素（N）は土壌窒素無機化の基質となり、炭素（C）は土壌微生物のエネルギー源となる。そして、C と N の量と比（C/N 比）は、土壌微生物による窒素変換のバランスを変化させる。森林生態系での土壌微生物への主要な有機物供給源は落葉と枯死根である。特に、冬季の土壌微生物の基質源として枯死細根の寄与は大きいと考えられている。そのため、冬季の気候変動による細根リターの増加は、土壌の窒素変換を大きく改変する可能性があるが、その影響は十分には分かっていない。細根リターの植物種が異なると、分解性（C/N 比）の違いを介して、凍結融解下の土壌中の窒素変換に影響する可能性がある。また、異なる植生下の土壌での土壌窒素変換は、冬季の気候変動に対して異なる応答を示す可能性がある。そこで本研究では、冬季気候変動を想定した凍結融解環境下において、細根リターが土壌の窒素変換に与える影響を明らかにすることを目的とした。具体的には、細根リターの植物種の影響と、異なる林分からの土壌による応答性の違いについて明らかにした。

調査は北海道東部に位置する京都大学北海道研究林標茶地区で行なった。標茶地区内のミズナラ二次林（下層植生：ミヤコザサ）に野外操作プロットを設置した。2014 年から 2015 年の冬にかけて、冬季気候変動による積雪量の低下を模倣した除雪実験を行なった。第二章では、野外操作プロットの基礎情報を示した。細根量は、生根ではミズナラがササよりも多いのに対し、枯死根ではササがミズナラよりも多かった。さらに、現地の地温変化を参考に予備培養実験を行い、第三章での室内実験の温度条件を設定した。異なる温度条件設定のうち、0 ～－5 ℃ での地温振幅条件下で窒素無機化速度が最も高まることを示した。

第三章では、細根リターの種類の違いが土壌の窒素変換に与える影響を明らかにするため、室内培養実験を行った。研究地の主要構成種であるミズナラとササを対象植物とした。土壌の凍結融解は細根リターの種類に関わらず、正味硝化速度を有意に低下させ、正味アンモニウム化速度および正味窒素無機化速度を有意に増加させた。また、ミズナラとササでは窒素無機化速度の促進時期が異なり、ササ細根は土壌凍結融解期間中に、ミズナラ細根は凍結融解後の融解期間中に窒素無機化を促進させた。凍結融解イベントによってササの細根リターから溶脱する溶存有機窒素（DON）はミズナラ細根リターよりも有意に多かった。また、ササ細根リターから放出される溶存有機炭素（DOC）と DOC/DON 比はミズナラ細根リターよりも有意に小さかった。これらのことから、C/N 比が低く、分解されやすい基質であるササ細根リターは、土壌微生物に利用されやすい溶存有機物を迅速に供給するため、土壌凍結期間中の窒素無機化が促進されたものと考えられた。一方、ミズナラ細根リターの添加では、凍結融解イベントによる物理的破砕等の作用によってリターの利用性が高まり、細根リターからの溶存有機物供給が増加することで融解期間中の窒素無機化が促進されたものと考えられた。さらに、細根リターの窒素無機化促進作用は凍結

融解によって増加し、その増加程度はミズナラ細根の方がササ細根よりも大きかった。このことから、凍結融解は分解性の低い細根リターの分解促進とそれに伴う窒素無機化の促進をもたらすと考えられた。

第四章では、異なる植生下の土壌における凍結融解に対する土壌窒素変換の応答を、野外操作実験によって検討した。野外操作プロットであるミズナラ林と、隣接するカラマツ林からそれぞれ土壌を採取した。各土壌には、両林分で共通の下層植生であるササ細根を添加した。この土壌試料を野外操作プロットの除雪区と対照区に埋設し、冬季の正味窒素無機化速度を測定した。除雪処理は土壌の最低地温を低下させ、凍結融解の振幅数を増加させた。除雪区の正味硝化速度と窒素無機化速度は、対照区よりも有意に低下した。一方、正味アンモニウム化は除雪区で対照区よりも有意に増加した。全 C 量、全 N 量、C/N 比に土壌間での有意差はなかったのにも関わらず、除雪区でのササ細根リターの添加に対する窒素無機化速度の応答は土壌間で異なっていた。すなわち、除雪区において、細根添加の無い土壌では、ミズナラ林土壌の方がカラマツ林土壌よりも窒素無機化速度が大きかったのに対し、細根を添加した土壌では、両土壌間の窒素無機化速度に有意差が認められなかった。これは、除雪区においてミズナラ林土壌は細根添加によって窒素無機化速度が低下したのに対して、カラマツ林土壌では細根添加による窒素無機化速度の変化は小さかったことに起因すると考えられた。細根添加に対する窒素無機化の応答の違いには、両林分下の土壌での溶存有機物の生成や利用に関する土壌微生物の機能の違いが関係していると考えられた。窒素無機化過程の基質である DON の正味消費速度は、ミズナラ林土壌の方でカラマツ林土壌よりも多かった。一方、土壌微生物のエネルギー源となる DOC の変化は、細根添加に対して土壌間で異なる応答を示した。細根添加によって、ミズナラ林土壌では DOC 生成量が低下したのに対し、カラマツ林土壌では増加した。このことは、溶存有機物の利用性や生成速度がカラマツ林土壌でミズナラ林土壌よりも高いことを示唆していた。

第五章では総合考察として、野外操作実験と室内培養実験の結果に基づき、気候変動による土壌の凍結融解イベントの変化が土壌の窒素変換に与える影響を議論した。野外実験で観測された冬季の DOC 生成速度の植生による違いは、土壌微生物による窒素消費への影響を介して、成長期における正味窒素無機化速度に対して植生間で対照的な変化を生み出すと考えられた。すなわち、冬季に DOC 生成量が低下したミズナラ林では成長期の正味窒素無機化速度が増加するのに対し、冬季に DOC 生成量が増加したカラマツ林では成長期の正味窒素無機化速度が低下すると予想された。また、室内実験で示された、凍結融解による細根リターの分解性の増加は、窒素無機化の促進とともに、両林分で土壌の C と N 蓄積量を減少させうると考えられた。

本研究から、冬季気候変動による凍結融解レジームの変化は、細根リターのような新鮮有機物の増加を介して、土壌の窒素変換に影響することが明らかとなり、その応答は植生によって異なっていた。また、細根リターの植物種の違いは、窒素無機化の増加タイミングが異なることで、土壌中の窒素変換に影響していた。以上のことから、冬季の気候変動が森林生態系の窒素動態に与える影響を評価する上で、森林の構成種や土壌微生物の機能の違いや、それらによる溶存有機物の動態を考慮することの重要性が示された。