The effect of freeze-thaw cycles on soil respiration and nitrogen dynamics as influenced by soil structure, aggregate size and water content

by

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Abstract

The role of soil structure and aggregation in influencing soil respiration and N dynamics in soils undergoing freeze-thaw cycles, as influenced by water content, is poorly known. Surface soil with a texture of sandy loam was collected from an agriculture field in Atlantic Canada. Two studies were undertaken. The first study determined the influence of soil structure (whole / crushed soil) and water content (35%, 60%, and 85% water-filled pore space) on soil respiration and nitrogen dynamics during freezing and thawing of the soil. The second study determined the role of different aggregate size fractions (0~0.25, 0.25~4, and 4~8 mm) on the freeze-thaw effect. The research found microbial metabolism is more limited by environmental conditions than by the substrate availability. Crushing altered structural characteristics and caused changes in substrate solubilization and / or microbial utilization of substrates during freezing and thawing. Furthermore, freezing and thawing did not influence the denitrification of the whole soil, but enhanced denitrification in soils where aggregates were crushed. At 60% WFPS, the interaction among aggregate crushing and freeze-thaw increased denitrification. Also, the impact of freeze-thaw was greater on soil respiration and N mineralization in medium size aggregates (0.25~4mm). Freezing and thawing improved denitrification in aggregates on all three size fractions. This project provided new information on the effects of freeze-thaw on soil carbon and nitrogen dynamics as influenced by soil structure and water content. This information will be critical in assessing the impact of climate change in soil carbon and nitrogen dynamics in temperate regions.

List of Abbreviations Used

CO_2 (carbon dioxide)

CR (crushed soil)

DMP (3, 5-dimethylpyrazole)

FT (freeze-thaw)

FTCs (freezing and thawing cycles)

NH_{4+}-N (ammonium nitrogen)

NI (nitrification inhibitor)

NO_{3-}-N (nitrate nitrogen)

N_2O (nitrous oxide)

WH (whole soil)

WFPS (water-filled pore space)

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Chapter 1. Introduction

1.1. Background

The freeze-thaw cycles of soil are thermal processes caused by the seasonal or diurnal temperature exchange between soil and atmosphere (Zhao et al., 2013). This phenomenon is widespread in high latitudes, high altitudes, and parts of temperate regions (Grogan et al., 2004). Soils that experience freeze-thaw account for about 70% of the total land area of the world — mainly in the northern hemisphere (Zhang et al., 2003). Increasingly, studies on soil N dynamics have shifted focus from the plant growing season to the overwinter period, focusing on the impact of winter processes on annual N budgets and losses to the environment.

Freeze-thaw cycles (FTC) are a vital driving force of soil N transformation in cold areas, which directly affect the turnover of soil N in the ecosystem (Joseph & Henry, 2008). In permafrost zones, low temperatures slow the decomposition of soil organic N, which leads to a deficiency of available N for plants; and hence, significantly limiting plant productivity in cold regions (Vitousek & Howarth, 1991). Furthermore, previous studies have shown that FTCs disrupt the physical structure and chemical properties of the soil, which affects not only microbial activity but also organic matter breakdown and N supply (Freppaz et al., 2007). The thawing of frozen soils can stimulate a burst in soil respiration and nitrous oxide emission – similar to the rewetting of dry soils (Congreves et al., 2018). There are two generally accepted mechanisms of this burst in respiration: one of them is

that freeze-thaw events destroy the physical structure of the soil aggregates and release protected substrates making them bioavailable (Soulides & Allison 1961; Denef et al. 2001). The second is the rupturing of microbial cell membranes, releasing energy and nutrient-rich cytoplasmic materials. The increase concentration of N-rich, bioavailable substrates stimulates microbial activity, and mineralization of N (Lipson & Schmidt, 2004; Sharma et al., 2006).

Global warming will decrease the distribution area of seasonal snow cover, altering soil temperature dynamics in the middle to high latitudes. Understanding how changes in FTCs caused by global warming influence the process of soil N transformation has become the focus of recent research. Improving our understanding of the impact of FTCs on soil N cycle processes will be critical to understand the implications of global climate change on soil N content and its impacts on air and water. An important first step is understanding the mechanisms of freezing and thawing impacts on soil N dynamics and their effects on plant available N. It is of considerable significance to provide information to farmers onto inform fertilization practices with the intention to increase N use efficiency, reduce soil N_2O emissions, and promote sustainable management of soil ecosystems.

1.2.Literature Review

This section summarizes the effects of freezing and thawing on soil physical properties (water content and aggregate size), soil respiration, soil soluble carbon, soil microbial activity, and two N transformation processes - N mineralization and denitrification.

1.2.1. Effects of freezing and thawing on soil physical properties

One of the primary impacts of FTCs is a change in soil structure. Soil aggregates, the association of soil particles into complexes comprised of sand, silt, clay, organic matter, and fungal hyphae, are the basic units of soil structure. Soil aggregation is an essential determinant of soil physical properties, indirectly affecting crop yields by regulating soil water, gas, temperature, and physical properties. The degree of soil aggregation and disaggregation is mainly related to soil type, organic matter content, water content, the initial size of aggregates, freezing temperature, and the number of FTCs (Koponen & Martikainen, 2004; Henry, 2007). Numerous studies have shown that the FTCs may reduce the stability of aggregates and lead to aggregate fragmentation (Oztas & Fayetorbay, 2003; Henry, 2007).

1.2.1.1.Effects of the freeze-thaw event and water content on soil aggregates

The active process in FTCs is the changing phases of the soil water, and therefore, the influence of freezing on aggregate disruption is closely related to the water content (Koponen & Martikainen, 2004). Due to its solute content, water in soil pores freezes below

0 °C. In response to water potential, free water that remains unfrozen in the soil will flow towards the frozen surface to form ice lenses (Hohmann, 1997; Bronfenbrener & Bronfenbrener, 2010). When liquid water transforms into a solid, the volume increases, hence, the expansion of ice crystals in the soil pores may disrupt the association between the particles, causing soil macro-aggregates to break apart (Radke & Berry, 1998). When the temperature rises above 0 °C, the ice begins to melt. Liquid water, together with the soluble components in the water, can be quickly expelled from the space left by the melting ice (Lehrsch et at., 1991; Oztas & Fayetorbay, 2003).

The physical effect of freeze-thaw will accelerate the breakup of large aggregates and increase the soil water permeability coefficient (Oztas & Fayetorbay, 2003). With the increase in water content, the water release and permeability of the soil increases accordingly since the act of physical disruption is enhanced with an increasing number of water-filled pores (Wang et al., 2020). Freezing and thawing results in increased soil porosity and changes in aggregate stability. The disruption of soil aggregates due to freezing and thawing increases with the greater soil water content. In addition, after melting, the increased amount of free water results in the nutrients that were trapped in the soil aggregates or adsorbed on the surface of the soil colloid being dissolved and lost as the water migrates (Wang & Bettany, 1993; Brooks et al., 1998).

Soil water content is also a critical factor in controlling soil denitrification. Soil water causes air to be displaced in soil pores and thereby impacting the aeration state of the soil (Sajedi et al., 2012). High water content will reduce the diffusion rate of oxygen in the soil,

which results in the formation of an anaerobic environment, conducive to denitrification (Shelton et al., 1999).

1.2.1.2.Effects on the freeze-thaw event on soil aggregate size

It is generally believed that the extent of soil disruption, as a result of freezing, is greater in larger aggregates than smaller aggregates (Edwards, 1991). Bullock et al. (1988) and Lehrsch et al. (1991) found that, after freezing and thawing, large sized aggregates were gradually broken down into small sized aggregates, while the small sized aggregates were transformed into medium sized aggregates (1 to 4 mm). The probable reason is that freeze-thaw can result in compression and thereby enhances the connection between particles (Lehrsch, 1998). In addition, van Bochove et al. (2000) found that successive FTCs significantly enhanced the denitrification in macroaggregates (0.25 to 5 mm). Among the range of aggregate size fractions studied (0.25 to 5 mm), denitrification activity was primarily associated with smaller size (0.25 to 2 mm) aggregates rather than larger size (2 to 5 mm) aggregates and increased after the freeze-thaw, which may relate to the smaller pores size and higher percentage of water-filled pores in smaller aggregates.

1.2.2. Effects of freezing-thawing on soil respiration

Soil respiration is the process of decomposition of organic substrates, which results in the release of carbon dioxide. It includes microbial respiration, root respiration, and soil animal respiration (Schlesinger & Andrews, 2000). Carbon dioxide can also be produced

as a result of the chemical oxidation of carbon-containing materials or dissolution of carbonate minerals.

Previous research has shown that during FTCs, soil respiration increases after thawing. This may be caused by the following two mechanisms:

i) The death of soil microorganisms as a result of freezing-thawing stress results in the release of cellular materials, allowing them to become available as carbon sources, stimulating the activity of surviving microorganisms (Feng et al., 2007).

ii) Soil aggregate disruption and release of sorbed and occluded organic matter under the alternating action of freezing-thawing, which stimulates the activity of micro-organisms. The result is increased respiration of soil microorganisms after a freeze-thaw event (Mohanty et al., 2014).

1.2.3. Effects of freezing and thawing on soil soluble carbon

Soluble carbon is closely related to the activity of microorganisms. It is a product of microbial metabolism, but also a substrate. The presence of soluble organic carbon provides a carbon source for microbial activity.

During a freeze-thaw event, soil organic carbon may experience the following processes:

i) with the extrusion of ice, the large soil aggregates are broken, and organic carbon protected by aggregate structure (occluded) is released, which is more conductive for use by microorganisms (Feng et al., 2007);

ii) the organic macromolecules, that are combined with/within soil aggregates, expand and contract as a result of changing temperatures and thus leading to the internal hydrogen bonds to break (desorption), which results in the release of smaller organic molecules. (Larsen et al., 2002);

iii) the migration of unfrozen free water to the ice will carry dissolved organics, which will result in greater concentration of dissolved organic carbon after melting (Slavik et al., 2012);

iv) the change of the water phase state causes the organic matters to shrink, causing the destruction of the bridging points with soil aggregates and increasing their solubility;

v) some microbial cells die in freezing, and release organic compounds (Grogan et al., 2004);

vi) the destruction of macroaggregates by freezing and thawing events will produce more microaggregates and organic colloids with larger specific surface area, which have a greater ability to adsorb organic materials, and then leading to the redistribution and dissolution of organic carbon in the soil solution during extraction (Mohanty et al., 2014).

1.2.4. Effects of freezing and thawing on soil microbial activity

When the soil temperature drops below freezing, some microbial cells die. The growth of intracellular ice crystals caused by the formation of ice crystals results in mechanical damage to cell membranes and organelles (Rivkina, 2000; Methé, 2005). At the same time, freezing decreases the water potential of the soil. The microorganism attempt to balance the water potential between the inside and outside of the cells by accumulating high

concentrations of solutes, primarily amino acids, in the cytoplasm to maintain intracellular water potential (Schimel et al., 2007). Upon rewetting the cytoplasmic solutes are released into the surrounding environment to balance changes in water potential at which time, they become solutes for microbial consumption. In the event of severe soil water stress, some microbes survive by dehydrating (Stark & Firestone, 1995), which reduces the fluidity of the cell membrane, and in severe cases, the cell wall is damaged (Finegold, 1996). Freezing can disrupt the cell membranes structure, including membrane organelles in cells such as mitochondria. The disruption directly affects or destroys physiological metabolic capability as it relies on the plasma membrane or membrane-bound organelles, disrupting in the generation, release and utilization of intracellular energy. It is well-known that the level of energy supply is closely related to the metabolic activity of microorganisms. When the energy supply is low due to freezing, the metabolic activity of microorganisms is reduced, and microorganisms may go dormant or die (Price & Sowers, 2004).

Soil microbial biomass is the total biomass of living microorganisms (Margesin, 2008). During soil freezing, low temperatures directly kill a considerable portion of the soil microorganisms, resulting in a rapid decline in microbial biomass, thereby increasing soluble organic components (Mikan et al., 2002; Jefferies et al., 2010). However, not all of the microbial biomass is killed during freezing. The surviving microbes, especially psychrophilic microbes, maintain their viability by utilizing substrates released from the disrupted soil aggregates, plant roots, and dead microbes (Herrmann & Witter, 2002; Koponen et al., 2006). In the early stages of soil thawing, the microbial biomass increases

sharply in response to the release of compatible solutes (amino acids) accumulated in the microbial cytoplasm during the freezing. The increase of effective substrates in soil stimulates the rapid growth and multiplication of microorganisms (Margesin, 2008; Jefferies et al., 2010). This increased biomass does not last long as the released substrates are rapidly consumed by the microbial activity in the soil producing a burst of CO_2 (Jiang et al., 2016).

1.2.5. Effects of freezing and thawing on nitrogen processes

1.2.5.1. Effects of the freeze-thaw events on net nitrogen mineralization

Nitrogen mineralization refers to the process of converting organic N into inorganic N in the soil (Schimel & Bennett, 2004) and is a result of the decomposition of organic substrates. The conversion of inorganic N to organic N is referred to as assimilation or N immobilization. The difference between total or gross N mineralization and immobilization is referred to as net N mineralization and is measured as the net accumulation of inorganic N in the soil. Hereafter N mineralization refers to the net effect of gross N mineralization and immobilization or net N mineralization.

Soil freezing intensity, freezing duration, and the number of freeze-thaw cycles have significant impacts on soil N mineralization. Zhang et al. (2011) found that severe freezing (-10 to -15 °C) induces a higher increase in N mineralization rate than mild freezing (-2 to -3 °C) in the soil. They speculated that, under mild freezing conditions, microorganisms are

able to maintain high level of activity and keep a steady N immobilization rate so that FTCs may not affect, or may even reduce, N mineralization rate. In addition, the extent of microbiological death during severe freezing, the number and activity of microbes surviving after thawing, are also critical determinants of subsequent rates of N mineralization. In the case of rapid and deep freezing, the number of surviving microorganisms is reduced, and the rate of N mineralization is decreased as the freezing temperature decreases (Hentschel et al., 2008). In research examining snow removal and overwinter in-situ sampling (Hentschel et al., 2009; Zhang et al., 2011), N mineralization rates increased significantly when freezing persisted for a long time (4 to 5 months) but was not impacted when the freezing time was short (3 months). However, this pattern was not observed in laboratory simulations experiments (Öquist et al., 2004; Clark et al., 2009), which may be caused by the differences in soil samples and experimental methods. The soil N mineralization rate increased when subjected to 1 to 2 freeze-thaw cycles under mild freezing conditions; however, when the number of freeze-thaw cycles was more than 2, the N mineralization rate decreased significantly (Zhou et al., 2011). It was suggested that this might be due to the long incubation period causing the accumulation of NH_{4+}, which inhibits further mineralization of soil organic N (Biondini et al., 1998). Hentschel et al. (2009) also found that the rate of N mineralization increased with the number of FTCs. In addition, the organic N content gradually decreased during multiple FTCs due to the consumption of mineralized N, hence, the rate of N mineralization showed a downward trend after a certain number of FTCs.

1.2.5.2.Effects of freeze-thaw events on denitrification

Denitrification is the reduction of NO_3^- and NO_2^- into gaseous NO, N_2O, and N_2 under anaerobic conditions by denitrifying microorganisms. This process is an essential step in the N cycle within the biosphere and is of great significance for regulating the reactive N pool (NO_2^-, NO_3^-, and NH_4^+) in soil ecosystems and balancing the N in the atmosphere (Bremner & Blackmer, 1980).

Enhanced soil denitrification capacity, especially N_2O production, as a result of FTCs has been observed in many studies (Christensen & Tiedje, 1990; Yanai et al., 2007). Röver et al. (1998) proposed that enhanced soil denitrification capacity is closely related to microbial activity in the FTCs by altering the soil microbial community. Mergel et al. (2001) found that the highest number of denitrifying bacteria were found in autumn, winter, and early spring, but at a relatively low level in summer, they concluded that this was related to microbial growth associated with FTCs. Secondly, even in the coldest months of the year, soil denitrification enzymes remain highly active (Pelletier et al., 1999). Increases in freeze-thaw intensity and frequency even promote nitrate reductase and nitrate reductase activity (Arias-Negrete et al., 2004).

The denitrification process depends mainly on three factors: soil aeration as influenced by water content, amount of available NO_3^-, and the quantity and quality of C substrate. In van Bochove et al. (2000), it was observed that the denitrification rate of soil that had undergone an FTC was 32% higher than that of unfrozen soil for the Kamouraska clay

(Orthic Humic Gleysol) slurries of Canada. They also found that the successive FTCs significantly enhanced the denitrification of macroaggregates (0.25 to 5mm). Among these range of aggregates, the denitrification activity associated with a smaller size (0.25 to 2mm) aggregates was higher, and the increase rate after the FTC was larger, which may be related to the higher water content of the smaller aggregates (van Bochove et al., 2000).

1.3.Knowledge Gap

Previous studies mainly focused on the impacts of FTCs on N_2O production (Chen et al., 2018; Pelster., 2019). There is relatively little research on how the FTCs affect N mineralization and denitrification processes. Moreover, little is known about the role that soil structure plays in the stimulation of biological activity upon thawing. Previous studies have shown that soil aggregate stability is affected to a varying extent by FTCs at different water contents. However, the changes in N dynamics resulting from FTCs with different water contents is not well understood. There are not many studies on the effects of freezing and thawing events on soil aggregates, and the studies on the different sizes of aggregates are rare. Among them, most studies focus on the effects of the FTCs on the stability of various aggregate size fractions. The role of FTCs on soil respiration and N dynamics from different soil aggregate size fractions, especially the microaggregates, is still poorly known.

1.4.Objectives

The main objective of this project is to investigate the role of FTCs on soil respiration and N dynamics as influenced by soil structure at different water contents.

To achieve the overall objective, the study would:

1) Determine the influence of water content in soil with different structures undergoing FTCs on soil respiration, N mineralization, and denitrification.
2) Evaluate the impact of successive FTCs soil on soil respiration and N dynamics as influenced by the soil structures and aggregate size fractions.

To examine the influence of soil structure, soils with intact soil structure (whole soil) were compared with soils where structure had been largely removed as a result of crushing of the soil aggregates (crushed soil). Structure was further considered by examining three different aggregate size fractions to determine how structure as influenced by aggregate size influenced the impact FTCs.

Chapter 2. Materials and Methods

2.1. Soil samples

For this study, 16.5 kg of surface soil samples was collected from a field cropped in an annual cropping system (corn-soybean) currently cropped to corn located at the Bio-Environmental Engineering Center, Bible Hill, Nova Scotia (45°23'02"N, 63°14'34"W) in May 2018. The climate is Dfb in the Köppen-Geiger system (immediately poleward of hot summer continental climates). Soils were air-dried at room temperature and all the visible roots and stones were removed prior to being passed through a 8 mm mesh sieve to retain macro-aggregate structure. The soil was a sandy loam textured acidic Podzol of the surface layer from 0 to15 cm. Chemical analysis of the soil indicated that it had 1.13% C and 0.20% N, giving a C/N ratio of 5.7; the pH was 5.63; the gravimetric water content of the following air-drying was 0.0137% w/w; and the concentration of NH_4^+-N and NO_3^--N were 3.04 and 5.40 mg kg^{-1} soil.

The soil samples were further processed for two following studies (Section 2.2.1 & 2.2.2). Two soil structure treatments would subject throughout the study 1 (Section 2.2.1) and study 2 (Section 2.2.2):

Whole soil: The air-dry soil that passed through an 8 mm mesh sieve.

Crushed soil: The air-dry soil that passed through an 8 mm mesh sieve was collected, and subsequently gently crushed using a pestle and mortar such that the crushed soil was able to pass through a 2 mm mesh sieve.

Three fractions of aggregates size fractions were separated from the whole soil for using in the aggregate fraction study (Section 2.2.2):

4 to 8mm fraction (Large): The air-dry soil that passed through 8 mm mesh sieve but not a 4 mm mesh sieve were collected for subsequent assay. The large size fraction accounted for 24.16% (± 2.38%) of the soil mass.

0.25 to 4mm fraction (Medium): The air-dry soil that passed through 4 mm mesh but not a 0.25 mm mesh sieve were collected for subsequent assay. The medium size fraction accounted for 62.38% (± 2.59%) of the soil mass.

0 to 0.25mm fraction (Small): The air-dry soil that passed through a 0.25 mm mesh were collected for subsequent assay. The small size fraction accounted for 13.57% (± 2.53%) of the soil mass.

2.2.Experimental design

2.2.1. Study 1: Influence of Water Content and FTCs on N transformations in Whole vs. Crushed Soils

The objective of this study was to the influence of water content in soil with different structures undergoing successive five FTCs on soil respiration, N mineralization, and denitrification. The study was a complete randomized design with five replications. Treatments included two soil treatments (whole soil and crushed soil, Fig.1), three water contents (35%, 60%, and 85% water-filled pore space), and two incubation conditions,

freeze and thaw cycles (FTCs) and a constant +5 ℃ condition (No-FT). Soil N processes were measured using sacrifices for each time step, and so a total of 72 sets (2 FT treatments * 2 soil treatments * 3 water contents * 6 time points at six-day intervals) established by placing 25 g of soil air-dry equivalent in 100 mL DigiTube plastic vials (SCP Science, Quebec) with five replications of each. All the samples were covered by parafilm (Bemis Company Inc., US) for moisture lost prevention.

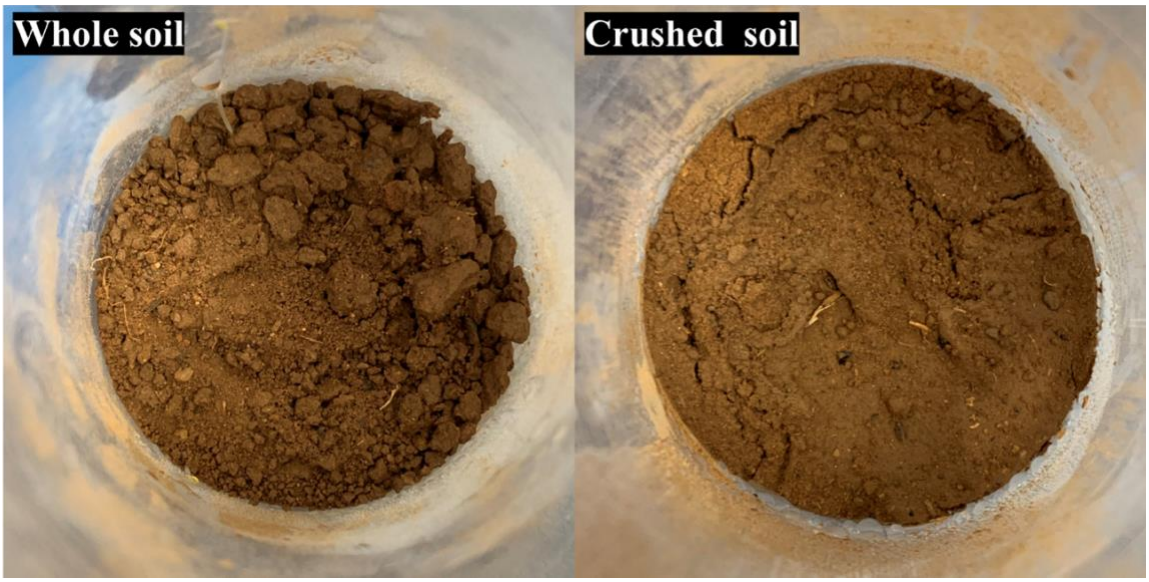

Figure 1:Twenty-five gram of soil air-dry equivalent placed in 100 mL DigiTube plastic vials, including the soil presence of aggregate (whole soil) and the soil in which the aggregates have been crushed (crushed soil).

Successive FTCs was used to achieve the gradually structure disruption of soil aggregates. In comparison with soil where aggregate were physically crushed to remove aggregate structure. Soil water-filled pore space was calculated based on a soil particle density of 2.65 g/cm$_3$ and an established bulk density of 1.3 g/cm$_3$ which was measured according to the method developed by Maynard & Curran (2008). The 60% WFPS was chosen as it is a water content typical of winter in Eastern Canada (Tatti et al. 2014). The

35% WFPS was selected to determine whether the insufficient water content influences the N processes under the effect of FTC. The 85% WFPS was chosen as this water content limits soil aeration.

2.2.2. Study 2: Effect of FTCs on soil respiration and N transformations in soil with/without aggregates and different aggregate size classes

The objective of the aggregate size fraction study was to determine the effects of FTCs on N processes in different aggregate size classes. A 0.25 to 4 mm was chosen as van Bochove et al. (2000) found that successive FTCs significantly enhanced the denitrification of this aggregate size fraction. A 4 to 8mm aggregate fraction was selected as the largest impact of freezing was observed in large aggregates of 4.75 to 9.5 mm (Edwards, 1991), and the proportion of aggregates that greater than 8 mm is small.

The study was conducted using a completely randomized design with five replications. The treatments in this study included multiple freeze-thaw treatments (FTCs) and constant +5 ℃ condition (No-FT), three aggregated size fractions (0 to 0.25 mm, 0.25 to 4 mm, 4 to 8 mm, Fig.2) plus two additional soil treatments (whole soil & crushed soil, Fig.1). The samples were wetted to 60% water-filled pore space (5.6791 g H_2O g^{-1} soil) and then subjected to five freeze-thaw cycles. Soil N processes were measured using sacrifices for each time step. A total of 60 sets (2 FT treatments * 5 soil treatments * 6 time points) was established by placing 25 g of soil air-dry equivalent in 100 mL DigiTube plastic vials (SCP Science, Quebec) with five replications. All the samples were covered by.

Figure 2: Twenty-five gram of soil air-dry equivalent placed in 100 mL DigiTube plastic vials, including three aggregate size fractions: 0 to 0.25 mm (small), 0.25 to 4 mm (medium), and 4 to 8 mm (large).

The < 0.25 mm soil was chosen as aggregates less than 0.25mm are defined as microaggregates. Microaggregates play a vital role in soil physical and chemical properties and biological properties.

2.3.Nitrification Inhibition Assay and Successive Freezing & Thawing Cycle (FTC)

A nitrification inhibition assay (Zebarth et al., 2019) was used to simultaneously measure microbial activity (soil CO2 production) and soil N processes (i.e. N mineralization and denitrification). Prior to experimentation, all samples were preincubated at 15 ℃ for ten days before each study. Deionized water was added to reach to 30% WFPS (2.8395 mL deionized water per 25 g air-dried soil) for the treatment of 35% WFPS and to 40% WFPS (3.7861 mL deionized water per 25 g air-dried soil) for the treatments of 60% and 85% WFPS. At the beginning of the pre-incubation period, the nitrification inhibitor, 3, 5-dimethylpyrazole (DMP), was added to reach a concentration of 200 mg kg^{-1} soil by adding 5 mg DMP per 25 g air-dried soil.

The soils were pre-incubated to enable the soil microorganism to acclimate to the higher incubation temperature, so that the process of denitrification could be activated (Drury, 2007). Secondly, pre-incubation minimizes the impacts of the "Birch effect", which is caused by a burst of microbial respiration (CO2 production) following the rewetting of an air-dry soil (Birch, 1958). Thirdly, a 10-days pre-incubation ensures that the DMP is inhibiting nitrification (Zebarth et al., 2019).

In total, the deionized water that was added per 25g air-dried soil was 3.3128 mL for 35% WFPS, 5.6791 mL for 60% WFPS and 8.0454 mL for 85% WFPS. At time = 0, the remaining deionized water was added to the soil to reach the proper water content and to deliver 100 mg kg^{-1} soil nitrate (as KNO3), that is, 2.5 mg per 25 g air-dried soil.

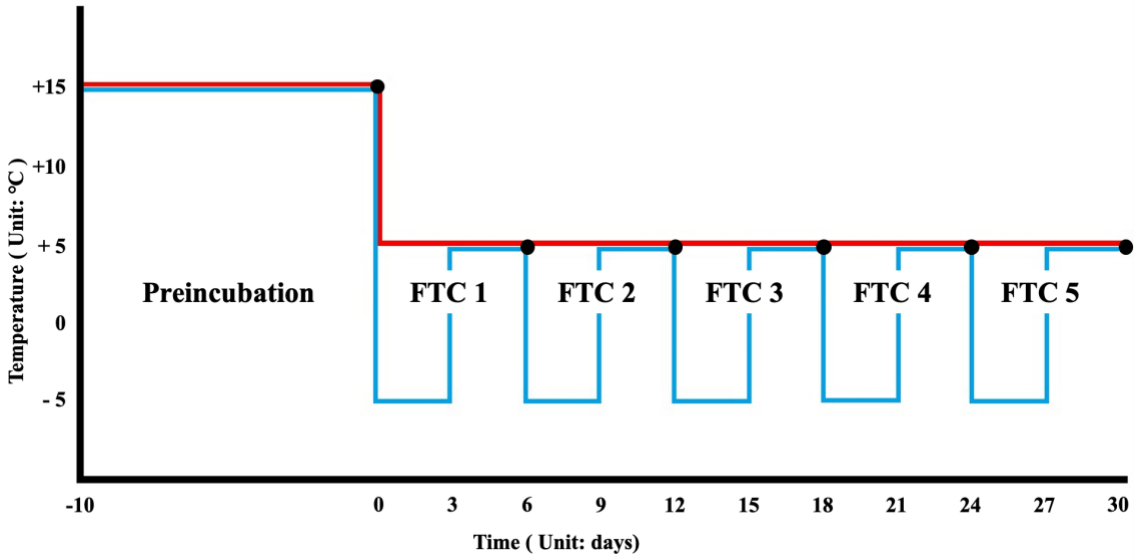

Figure 3: Scheme of successive FTC assay. Sample are preincubated for ten days and then subjected to 5 FTCs (3-days freezing at -5 ℃ and 3-days thawing at +5 ℃). Crushed soil samples and whole soil samples will go through a total of 5 FTCs (blue line), and a No-FT Group (red line) would set up for each treatment. Black dots mean the sampling time points.

The soil samples were incubated using Precision™ low temperature BOD refrigerated

incubator (Thermo Scientific™, US). A FTC was defined as a 3-days period at −5 °C followed by a 3-days period of thawing at +5 °C. A total of 5 FTCs were applied in each experimental group. A No-FT Group (control) was incubated at constant +5 °C. Successive FTCs treatment was applied to two following studies (Section 2.2.1 & 2.2.2). The experimental setup is shown in Fig.3. The chosen temperatures relate to soil temperatures recorded in eastern Canada, which typically range from −8 to +15 °C between November and April (Wertz et al., 2016).

2.4.Soil analysis

2.4.1. Soil respiration

To assess the activity of soil microorganisms, soil respiration was measured by trapping CO_2 from experimental units during each FTC in 10 mL of 0.5 M KOH solution. Trapping in alkali allowed for continuous collection of CO_2 during the incubation period and did not result in the accumulation of CO_2 in the headspace of the incubation vessel. The measurement of electrical conductivity was used to quantify the carbonate content of the solution as it is markedly faster than titration, and less hazardous compounds were used (Wollum & Gomez 1970; Tongway et al., 2003). The reactions for CO_2 trapping in an alkali solution is shown by:

$$2KOH + CO_2 \rightarrow K_2CO_3 + H_2O$$

Electrical conductivity (EC) was measured as a proxy for the amount of salt (K_2CO_3)

produced using a FiveEasy Benchtop FP30 Conductivity Meter (Mettler Toledo, Switzerland). The amount of CO_2 generated was calculated according to the formula (Wollum & Gomez 1970):

$$\text{Amount of } CO_2 \text{ in mg} = [(EC_{raw} - EC_{sample}) / (EC_{raw} - EC_{sat})] * (\text{trap capacity})$$

where ECraw is the electrical conductivity of pure 0.5 M KOH, ECsat is the electrical conductivity of 0.25 M K_2CO_3, ECsample is the electrical conductivity of the trap associated with a sample, the trap capacity is the maximum amount of CO_2 that the KOH trap can absorb.

The alkali traps were changed after each FTC to measure the cumulative amount of CO_2 emitted over one FTC (6 days). CO_2 production over all five FTCs was obtained by summing the CO_2 trapped for individual experimental units for the same treatment from FTC 1 to FTC 5.

2.4.2. N mineralization and Denitrification Measurement

Nitrogen mineralization and denitrification were measured simultaneously using the nitrification inhibition assay method described by (Zebarth et al., 2019). Since NH_4^+ conversion to NO_3^- was blocked by the addition of an inhibitor, the increase in the concentration of NH_4^+-N was used as a measure of N mineralization, and the decrease in the additional NO_3^--N concentration was used as a measure of denitrification. The C:N ratio of the soil 5.7:1 suggests that net mineralization would predominate, and immobilization of inorganic N would not be significant. Soil mineral N concentrations were determined on destructive samples following the method of Maynard et al. (2008), as modified by Zebarth et al. (2019). Twenty milliliters of 0.5 M K_2SO_4 were added to each soil sample vessels to

establish a 1:2 v/v soil: K_2SO_4 ratio, and then caped and shaken for one hour on a lateral shaker. Soil slurries were allowed to settle for 1 hour and filtered by 2.5 μm qualitative paper (Whatman, CAT#1005-110) to collect the supernatant, which were stored at -20 ℃ until analysis. NH_4^+-N, NO_3^--N concentrations were measured by colourimetric analysis using a Technicon AutoAnalyzer II system (Seal analytical, UK), following the protocols in the Technicon Industrial Method #98-70W for NH_4^+-N, and Technicon Industrial Method #100-70W for NO_3^--N (Technicon Industrial Systems, 1978a; Technicon Industrial Systems, 1978b).

2.5.Statistical analysis

The study 1 (Section 2.2.1) was a 3-factorial experiment, i.e. i) incubation condition – two treatments (FTC and no-FTC), ii) soil treatments – two treatments (whole and crushed aggregates), and iii) water contents – three treatments (35%, 60% and 85% WFPS) for a total of twelve treatment combinations. the study 2 (Section 2.2.2) was a 2-factorial experiment, i.e. i) incubation condition – two treatments (FTC and no-FTC), and ii) soil treatments – five treatments (whole and crushed aggregates, three aggregate size fractions) for a total of ten treatment combinations. Data were analyzed using SAS 9.4 (SAS Institute, US) for the 3-way analysis of variance (ANOVA) to test for significance at 95% confidence level. The residues were tested to check basic assumption of independence, normality and constant variance. For the assumption that was violated, data transformation was performed, i.e. in the study 1, the soil respiration was transformed using negative square root, and the

values were transformed back for data interpretation. The ANOVA was performed using Proc Mixed model with fixed factors in SAS 9.4. Soil respiration, N mineralization and denitrification were test as responses at both studies, and incubation conditions, soil treatments, water content, and their interactions were modeled as fixed effects. Treatment means were separated using Tukey pairwise comparisons when the ANOVA indicated a P-value < 0.05. Regression analysis to identify the main trends of some of the variables over time were carried out by SAS 9.4 (SAS Institute, US). Graphs were plotted using Microsoft Excel 365 (Microsoft Corporation, US).

Chapter 3. Results and Discussion

3.1. Influence of Water Content and FTCs on N transformations in Whole vs. Crushed Soils

3.1.1. Soil respiration

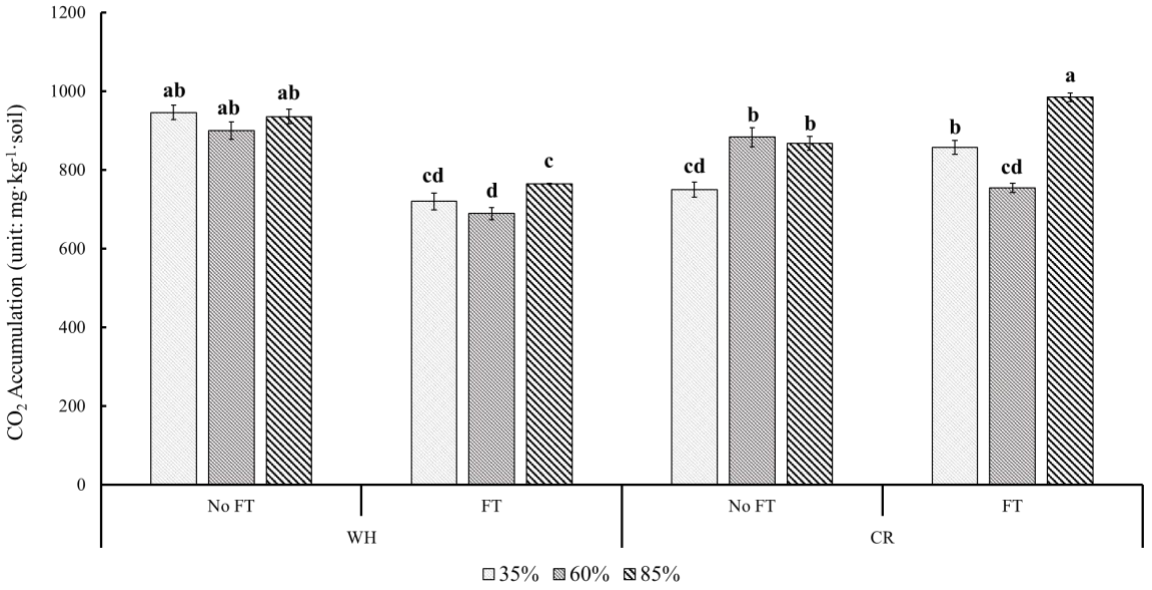

Figure 4: Soil respiration (CO_2 production; mg·kg$_{-1}$·soil) over five FTCs as influenced by water-filled pore space (WFPS) and the presence of whole soil (WH) or soil in which the aggregates have been crushed (CR). The results for soils that have been subjected to five freeze-thaw cycles (FT) are compared to soils maintained at 5 ℃ (No FT). The three-way interaction treatments that do not share a letter are significantly different. Mean value and standard error of the mean are presented.

Physical and chemical properties such as temperature, water content, organic C content and soil porosity, are factors that may affect soil respiration (Han et al., 2007; Zheng et al., 2009; Zhou et al., 2016), and the influence of soil moisture, soil temperature and soil structure on soil respiration are often interrelated (Matzner & Borken, 2008). In this study,

soil structure and water content were tested as factors influencing the impact of freezing and thawing on soil respiration. The CO_2 emissions from were measured by trapping in an alkaline solution. The results revealed that soil structure and water content had different effects on soil respiration. WFPS, the presence of aggregates and freezing and thawing significantly influenced on CO_2 production when summed over the five FTCs (Fig.4).

After successive FTCs, the whole soil had significantly lower cumulative respiration than the soil that was maintained at 5 ℃, regardless of water content (Fig.4). The cumulative respiration of soil decreased by 22% on average after freezing and thawing, indicating that respiration in the whole soil was negatively impacted as a result of freezing and thawing. The lower respiration suggests that freezing and thawing inhibited the activity of soil microorganisms and/or the supply of substrate to them. Lomander et al. (1998) also gave the similar the observations that the soil incubated under FTCs showed a lower CO_2 evolution that incubated at a constant 5 ℃. The possible explanation of this phenomenon is that the 5 ℃ was still a relatively cool temperature, and three days was an insufficient timeframe for microbial activity to be restored. The soils held at 5 ℃ provided a greater amount of heat units (degree day above zero) to support microbial metabolism, a total of 150 degree-days above zero (30 days * 5 ℃) compared to only 75 degree-days in the FTC treatments (15 days * 5 ℃). Microorganisms have specific temperature ranges for normal growth and reproduction. The lower the temperatures, particularly those below freezing, inhibit the activity of the microorganisms (Pietikäinen et al., 2005). The enzyme activity decreases with the temperature, slowing down microbial metabolism and the growth and

reproduction of microorganisms (Koch et al., 2007). Kurganova et al. (2007) found that CO_2 emissions from the soil incubated at -5 ℃ was about 5 to 20% of soil that incubated at 10 ℃, which is extremely low. In our study, microbial activity could not recover from the low temperatures in the FTC treatments over the three days thaw at 5 ℃ to offset the CO_2 production in the whole soil that remained at 5 ℃ over the six days of incubation.

The influence of water content on soil respiration was even more dramatic when the aggregates were crushed, and the soil was submitted to FTCs (Fig.4). Water content did not have a significant impact on soil respiration in whole soils when the temperature was maintained at 5 ℃. However, when the aggregates were crushed, water content did have a significant impact on soil respiration (Fig.4). For the soil maintained at 5 ℃, by comparing the soil respiration of whole soil and crushed soil, it can be concluded that if any substrate was released by crushing the soil aggregates, it did not promote a significant increase in soil respiration at higher moisture contents. Crushing soil aggregates decreased soil respiration at 35% WFPS (750 $mg \cdot kg^{-1} \cdot soil$) compared to whole soil (945 $mg \cdot kg^{-1} \cdot soil$) indicating that the availability substrates were not increased by crushing to affect increased soil respiration.

Based on the electrical double layers that forms at the surface of charged soil particles, the water ion can be generally classified as bound water associated with the particle surfaces and free water. The bound water, also known as hygroscopic water, is present closest to soil surfaces (Ross, 1978). Hydroscopic water molecules and hydrated ions are arranged very closely to surfaces, the dissolution capacity is weak, and no freezing occurs, even if the

temperature drops below 0 °C (Anderson & Tice, 1971; Zilberbrand, 1997). Free water in the soil is divided into capillary water and gravitational water. Capillary water, formed by the adhesion between water molecules and the surface of soil particles and the surface tension of water, is mainly held in the micropores of the soil (Baker & Frydman, 2009). When the soil moisture exceeds the field capacity, excess water (gravitational water) will move downward in soil macropores due to gravity (Bouyoucos, 1921). Simply put, as the exposed surface area (soil specific surface area) of the soil increases, the more bound water it can contain (Tall, 2019). As the size of soil aggregates increases, the number of micropores in the soil decreases and the number of macropores increases.

Crushing soil aggregates increases the specific surface area of soil. Therefore, the ratio of free water to bound water in whole soil will be greater than that in soil where aggregates have been crushed. The mobility of bound water is not as great as that of free water, and the substrate diffusion efficiency is reduced. Although the concentration of substrates available to microorganisms in the crushed soil may have increased slightly, the difficulty of obtaining substrates by microorganisms may have also increased, resulting in no significant increase in soil respiration. This may also explain why the soil respiration of crushed soil with 35% WFPS was significantly lower than that of crushed soil with 60% and 85% WFPS. In the crushed soil with 35% WFPS, the ratio of free water to bound water was smaller, and the free water content was insufficient to support the overall diffusion of substrates in the soil.

Due to the FTCs, the respiration of whole soil at 85% WFPS was significantly higher

(764 mg·kg^{-1}·soil) than that of whole soil at 60% WFPS (689 mg·kg^{-1}·soil). Some literatures suggest that that high water content will have a much greater impact on soil aggregate disruption and release more substrates to microorganisms (Wang & Bettany, 1993; Brooks et al., 1998; Sajedi et al., 2012). However, respiration in soil where the aggregates were crushed at 60% and 85% WFPS in the No-FT Group were not significantly different from that of whole soils in the No-FT Group, which suggests that crushing alone did not result in a release of the substrate, but only when subjected to FTCs. One possible explanation is that soils with higher water content need more time to freeze deeply, giving the microbes enough time to respond to the stress of freezing by accumulating compatible solutes. The response resulted in less microbial death and greater microbial activity in more moist soils, allowing them to recover faster and produce more CO_2 than those grew in low-water soils. Also, higher water content allows for greater diffusion of substrates to the microorganism. At lower water contents, diffusion may limit microbial activity. The possible reason why the respiration of whole soil with 35% WFPS was not significantly lower than that of whole soil with 60% WFPS is that the ratio of free water to bound water in whole soil with 35% WFPS was lower than that in whole soil with 60% WFPS. In soil, the freezing point of the bound water is much lower than that of free water, so the time it takes for the 35% WFPS soil to freeze completely was not less than that of the 60% WFPS soil.

There was an interaction between freezing-thawing and the presence of soil aggregate as indicated by the increased soil respiration in the FT group of crushed soils with 35% and

85%WFPS, but not whole soils. One of the possible explanations for this observation is that the crushing operation destroyed the soil aggregate structure and thus exposed the substrate protected by physical structure, and increases the surface area of aggregates, which provide microorganisms with more living spaces. In addition, because the water potential of unfrozen water is higher than that of ice, freezing and thawing promoted the water mobility in the soil at the microscopic level, which facilitates the diffusion of substrates and gives microorganisms access to substrates. Their interaction increases the availability of soluble organic matter in the soil, resulting in higher CO_2 production.

In the presence of aggregates, freezing and thawing did not result in a significant release of substrate and therefore the smaller number of heat units for microbial metabolism resulted in less CO_2 relative to the +5 ℃ environment. When the aggregates were crushed, freezing and thawing did result in a release of substrates that the microbes could access and thus a burst of respiration that exceeded the amount when kept at 5 ℃. Crushing the aggregates alone was not sufficient to release this substrate at low water content but did result in increased respiration at higher water contents.

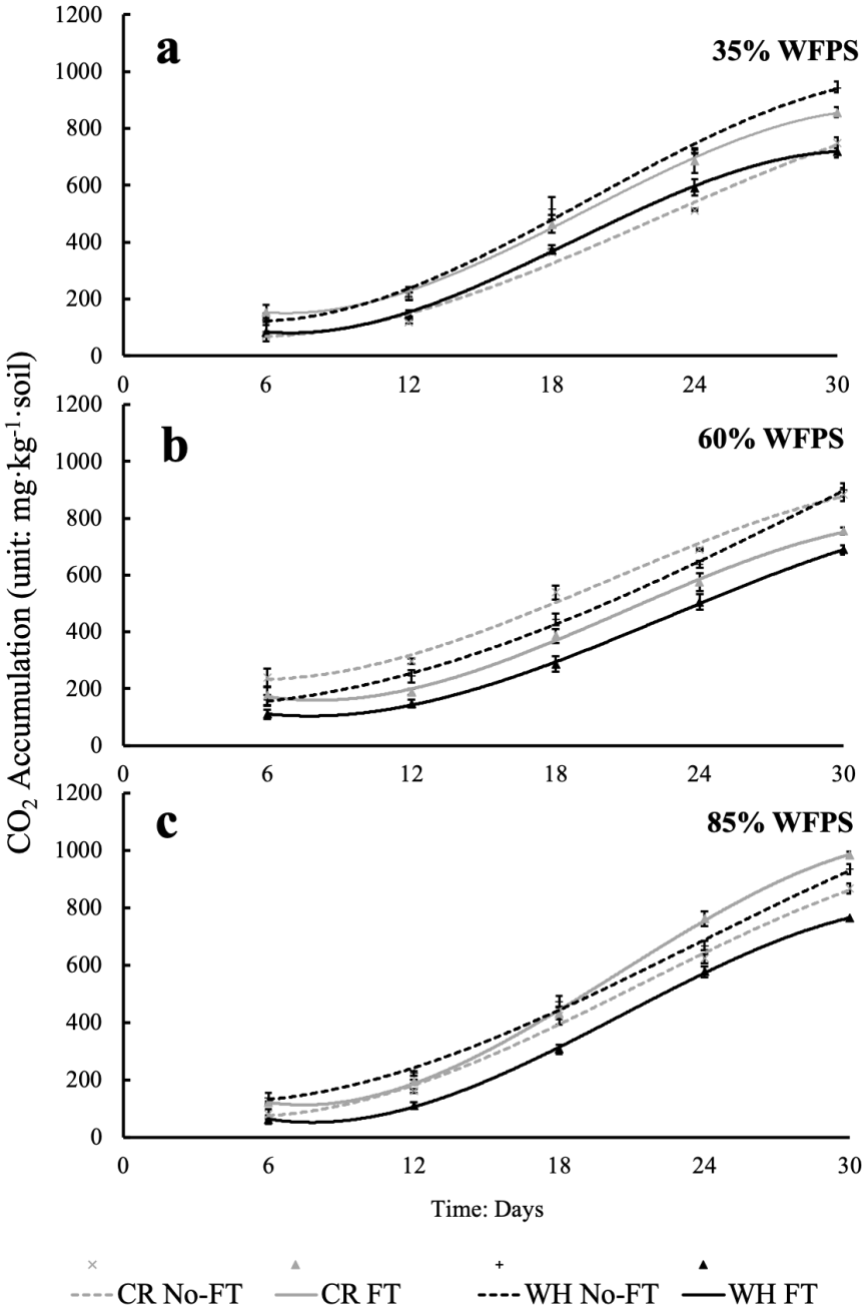

Figure 5: a) Curves of CO_2 accumulation in different soils structure over time at water content of 35% WFPS; b) Curves of CO_2 accumulation in different soils structure over time at water content of 60% WFPS; c) Curves of CO_2 accumulation in different soils structure over time at water content of 85% WFPS (unit: mg·kg$_{-1}$·soil), where FTC means the number of freezing and thawing cycles; CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; No FT means the treatment that maintained at 5 ℃ environment; FT means the treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

The temporal pattern of CO_2 accumulation over the five FTCs (30 days) reveals a relatively consistent pattern of CO_2 accumulation with lower rates of CO_2 emission during the first two FTCs (day12), indicating that the respiration of the microbial community in the early stage was not high. This may be because the psychrophilic soil microbial community may have taken time to establish due to the preincubation of the soil at 25 ℃ (Kotsyurbenko, 2005). After the first FTC, the rate of CO_2 accumulation is relatively consistent. In the soils at 35% WFPS (Fig.5a), the respiration of the soils that remained at 5 ℃, after a slow recovery in the early period, rose steadily in the later period, indicating that the soil respiration rate was stable. In contrast, the respiration of the freeze-thaw treated soil began to slow down during the fifth freeze-thaw cycle. For the soils at 60% WFPS (Fig.5b), it can be seen that there was a visible gap of soil respiration between crushed soil and whole soil held at 5 ℃ in the early stage, and the gap narrowed over time. In terms of soils at 85% WFPS (Fig.5c), the respiration rate of freeze-thaw treated soils was higher than that of soils that have not undergone freezing and thawing. However, similar to the freeze-thaw treated soil at 35% WFPS, the soil respiration rate slowed during the last freeze-thaw cycle.

3.1.2. Nitrogen mineralization

This study determined the effect of different water contents, soil structure, and freeze-thaw events on soil N mineralization. Mineralized N was calculated as the sum of NH_4^+-N accumulation over five FTCs.

Table 1
The amount of N mineralized of soil as affected by different soil structure, freezing and thawing, and separate water content (unit: mg·kg^{-1}·soil). Means followed by different uppercase letters indicate significant FTC effects; means followed by lower case letters indicate significant WFPS effect.

	FTC		WFPS		
Structure	No FT	FT	35%	60%	85%
Whole	3.76±0.50 ^{B}	5.51±0.45 ^{A}	4.29±0.33 ^{b}	2.77±0.36 ^{c}	6.85±0.34 ^{a}
Crushed	3.94±0.44 ^{B}	3.61±0.40 ^{B}	4.13±0.49 ^{bc}	2.92±0.54 ^{bc}	4.28±0.43 ^{b}
Structure		*			
WFPS		*			
Incubation		*			
Structure X WFPS		*			
Structure X Incubation		*			
WFPS X Incubation		n.s.			
Structure X WFPS X Incubation		n.s.			

Mean values ± standard error are shown;
* significant at 5% level;
n.s. no significant difference

There was a significant impact of the presence of aggregates, WFPS and FTCs on N mineralization. There was a significant interaction effect between the presence of aggregates and WFPS and between the presence of aggregates and FTCs. Water content only influenced the N mineralization in soils where the aggregates were intact (Table 1). The whole soil resulted in increased N mineralization only at 85% WFPS, but not at 35% and 60% WFPS. There was also an increase in N mineralization in whole soil only when

soils were exposed to FTCs.

As for the soils held at the 5 ℃ environment (No-FT), there was no significant difference between whole soil (WH) and crushed soil (CR) in N mineralization (Table 1), indicating crushing aggregates did not significantly affect the N mineralization of the soil. However, after freeze-thaw treatment (FT), the N mineralization of whole soil was enhanced by about 50% relative to the soil held in 5 ℃. By contrast, the N mineralization of crushed soil was not affected by successive FTCs. Previous studies have also shown that freezing and thawing could significantly promote the N mineralization rate by 11 to 300 % (Herrmann & Witter., 2002; Freppaz et al., 2007). Those studies also suggested that the main reason for the increase in soil N mineralization rate during freezing and thawing is the increase of microbial availability of soil organic nitrogen resulting from biological death and/or soil structure fragmentation (Herrmann & Witter., 2002; Freppaz et al., 2007). Here, complete fragmentation of the aggregates by crushing did not result in enhanced N mineralization as a result of FTCs at any of the three water contents examined, suggesting that the presence of aggregates is an important aspect of FTC-induced N mineralization, but FTC does more than simply disrupt the aggregates.

Soil that remained at 5 ℃ did not show a significant difference in the N mineralization as a result of crushing the soil aggregates, hence, the organic nitrogen exposed during the crushing process did not support enhanced N mineralization. Freezing and thawing also did not significantly affect the N mineralization of crushed soil. In other words, enhanced mineralization required both the presence of soil aggregates and freezing and thawing

cycles, which may be because the increase in the exposure of soil particles and the elimination of large pores resulted in interfered with the influence of freezing and thawing on the microbial population or the mechanism of substrate solubilization.

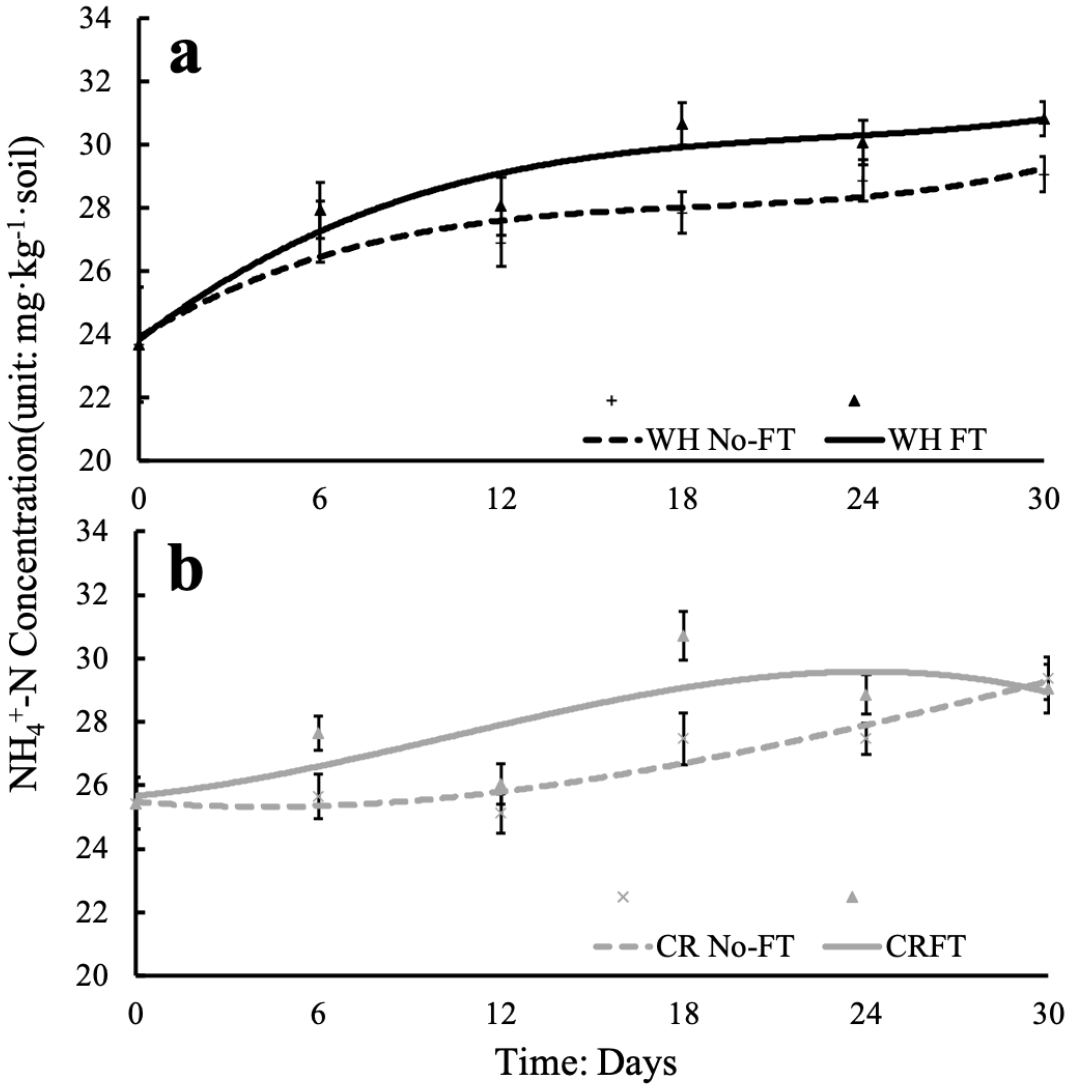

Figure 6: a) Curves of NH_{4+}-N concentration in whole soils with different FTC treatments over time; b) Curves of NH_{4+}-N concentration in crushed soils with different FTC treatments over time (unit: mg·kg$_{-1}$·soil), where FTC means the number of freezing and thawing cycles; CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; No FT means the treatment that maintained at 5 ℃ environment; FT means the treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

Although crushing did not show an effect on N mineralization during the incubation period of freezing and thawing, we can still find some information in Fig.6. In the soil where the aggregate structure was maintained and held at 5 ℃ (WH-No FT) changes in NH_4^+-N concentration occur at a slow but consistent rate (Fig.6a). In contrast, NH_4^+-N level in soils where the aggregate structure was maintained and was undergoing freeze-thaw (WH-FT) increases rapidly in the first three FTCs (day 0 to 18), while little to no increase in the final two cycles — observations that were similar to that of Hermann & Witter (2002) where the flush of N mineralization appeared briefly in the first four FTCs. This may be because most of the organic nitrogen was quickly released in the first few FTCs, and as the number of freeze-thaw cycles increased, the amount of available substrates and nutrients also decreased, resulting in a decline in the release of NH_4^+ after freeze-thaw (Hentschel et al., 2008). Similar to the whole soil held at 5 ℃, the NH_4^+-N concentration of soils where the aggregates were crushed, and the soil held at 5 ℃ (CR-No FT), also changed slowly but consistently over time (Fig.6b). The difference was that in soils where the aggregates were crushed and maintained at 5 ℃ had an elevated initial NH_4^+-N concentration but very little subsequent increase in NH_4^+-N level. It appears that the crushing of the soil aggregates may have resulted in increased N mineralization. The elevated NH_4^+-N concentration may reflect aggregate disruption-induced N mineralization during the pre-incubation period. The NH_4^+-N concentration was higher in crushed soil than in whole soil at time zero, which implicated the substrate of crushed soil might be released during pre-incubation and were subsequently not available to be mineralized later. The NH_4^+-N concentration of crushed

soil that has undergone freeze-thaw treatment (CR-FT) fluctuates greatly over time due to the effects of successive FTCs.

It is generally believed that soil moisture content is a key factor influencing the mechanism of freezing and thawing effects on soil N mineralization (Freppaz et al., 2007). However, there was no significant interaction between WFPS and FTCs (Table 1). In our study, the presence of soil aggregates was a more critical factor, as indicated by the Structure X WFPS and Structure X FTCs interactions. Among the whole soil, the soil with a water content of 60% WFPS had the lowest in N mineralization, which indicates that under the conditions of this study, low water content promoted N mineralization of the whole soil as compared with intermediate water contents, probably because of its higher air permeability. However, the phenomena that 85% WFPS also promoted N mineralization of the whole soil is surprising and unexplained. Crushing had no significant effect on N mineralization in soils with lower water content (35% and 60% WFPS). However, it significantly reduced the N mineralization of soils with high water content from 6.85 to 4.28 $mg \cdot kg^{-1} \cdot soil$, indicating that the promotion effect of high water content on N mineralization was inhibited by crushing treatment. This observation may have been a result of the crushing treatment, which destroys the structure of the aggregates and thereby eliminating the air bubbles and the living space for microorganisms in the large aggregates, resulting in lower N mineralization of crushed soil than that of whole soil at 85% WFPS.

3.1.3. Nitrate and Denitrification

The denitrification was determined by measuring the consumption of NO_3--N in soils treated with a nitrification inhibitor over the course of five successive FTCs. Freeze-thaw did not affect the denitrification in soils where the aggregate structure was maintained at any of the three water contents studied but significantly enhanced the denitrification in soils where aggregates had been crushed. Aggregation did not affect denitrification, but water content and FTC treatment significantly influenced denitrification. The three-way interaction between aggregates, WFPS, and FTC on denitrification was significant.

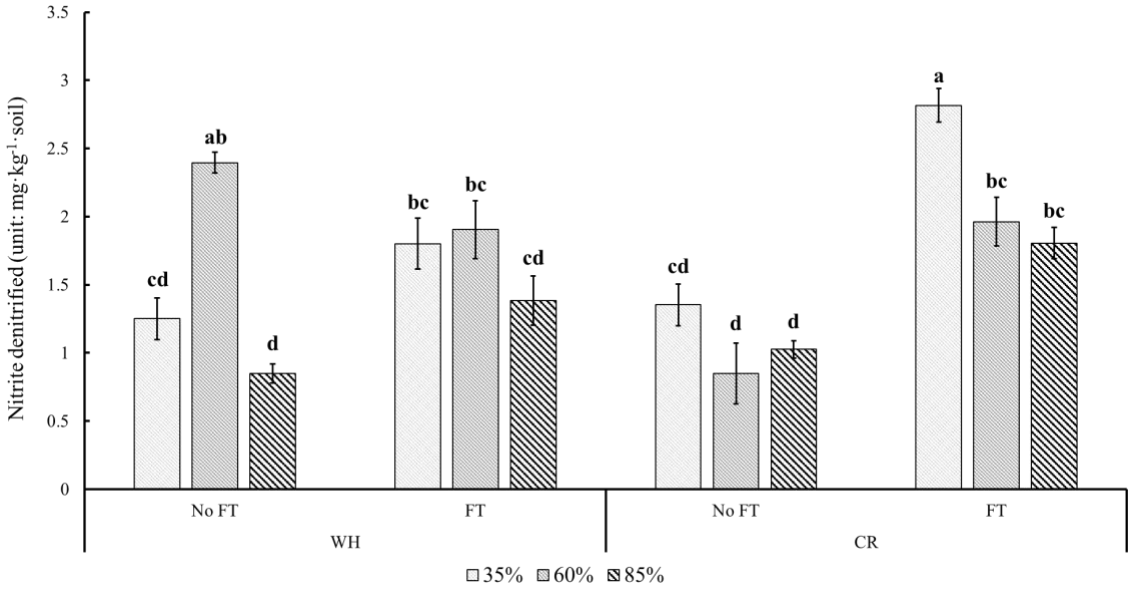

Figure 7: The amount of nitrate denitrified on study 1 (unit: mg·kg-1·soil), where CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; No FT means the treatment that maintained at 5 ℃ environment; FT means the treatment that experienced successive freezing and thawing cycles. The treatments that do not share a letter are significantly different. Mean value and standard error of the mean are presented.

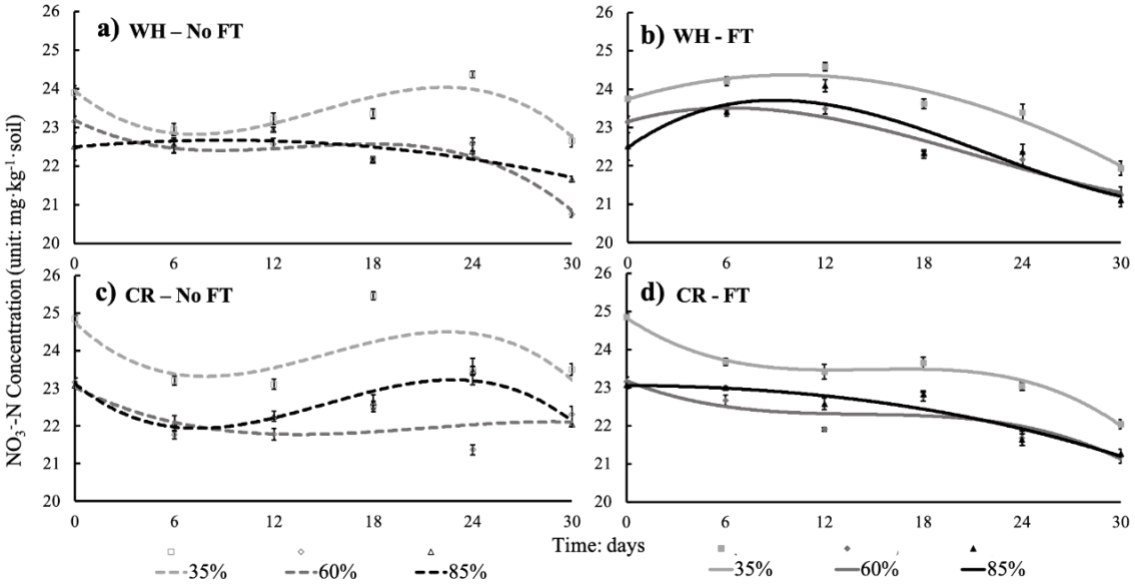

Figure 8: a) NO_3--N concentration of whole soil held at 5 ℃ at three different water contents held at 5 ℃ (WH-No FT); b) NO_3--N concentration of whole soil subjected to five freeze/thaw cycles at three different water contents (WH-FT); c) NO_3--N concentration of soil in which aggregates have been crushed held at 5 ℃ at three different water contents over time (CR-No FT); d) NO_3--N concentration of soil in which aggregates have been crushed and subjected to five freeze/thaw cycles at three different water contents (CR-FT) (unit: mg·kg-1·soil), where FTC means the number of freezing and thawing cycles; CR means the soil treatment that the aggregates were crushed; WH means the soil the aggregates remained intact; No FT means the treatment that maintained at 5 ℃; FT means treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

In soils where aggregate structure was maintained, FTCs effect did not affect the denitrification (Fig.7). However, for the whole soil held at 5 ℃, the denitrification at a water content of 60% WFPS (2.40 mg·kg-1·soil) was greater than that of 35% WFPS (1.25 mg·kg-1·soil), which is attributed to better aeration conditions of soil at 35% WFPS. Better aeration conditions resulted in high oxygen availability which would inhibit denitrification. Denitrification is an anaerobic respiratory process, which is inhibited as the oxygen concentration in the microenvironment increases (Li et al., 2020).

In soil at 60% WFPS at constant 5 ℃, denitrification was significantly reduced in crushed soil aggregates relative to the whole soil, 2.40 v.s. 0.85 mg·kg^{-1}·soil — a rate that is similar to that of soils at 35% WFPS and 85% WFPS (Fig.7). The crushing of aggregates would result in greater exposure to the atmosphere, and more inhibition by O_2. The denitrification of crushed soil with all three water contents were enhanced after freeze-thaw treatment. In this study, the crushing operation destroyed the soil structure, grinding the aggregates into particles smaller than 2 mm. Van Bochove et al. (2000) found that smaller-size (0.25 to 2mm) aggregates had higher denitrification activity after freeze-thaw. They attributed this phenomenon to the higher water content of smaller aggregates. Because the soil water content in this study was determined based on the conclusion of van Bochove et al. (2000), it can be speculated that the crushed soil or smaller aggregates have higher denitrification rates because they have higher bound-water/free-water ratio. For the same mass of soil, smaller size aggregates have a larger specific surface area. The bound water adsorbed on the surface of the aggregate was frozen into an ice film during the freezing period, and forming a closed anaerobic space (Wang et al., 2004), which created conditions conducive to denitrification.

The trend in NO_3^--N concentration of whole soil and crushed soil at different water contents over successive FTCs is shown in Fig 8. In general, the NO_3^--N concentration of soil at 35% WFPS was always the highest, while the overall difference of NO_3^--N concentration was smaller between 60% and 85% of soil. At 35% WFPS there was both positive and negative fluctuations in NO_3^- concentration. The increases in NO_3^-

concentration suggest that nitrification was not completely inhibited. Zebarth et al., (2019) indicated that the nitrification inhibitor (DMP) appears to be less effective at lower water content. This may reflect poor distribution of the nitrification inhibitor in soil. Because 22.58 mg·kg^{-1}·soil of NO_3^--N was added at the time zero, the soil at 5 ℃ gave a large decrease of NO_3^--N concentration in the first freeze-thaw cycle by additional reaction substrate. The NO_3^--N concentration of soil at constant 5 ℃ occurred fluctuations after the third FTC, which might be related to failure of nitrification inhibitor. Zebarth et al., (2019) found the inhibitory effect of DMP began to decline after 2 to 4 weeks of preincubation at 25 ℃. As a comparison, the changes in the NO_3^--N concentration of the two soils after the freeze-thaw treatment with FTC were significantly different. Crushed soil's NO_3^--N concentration (CR-FT) decreased steadily with the increase of the number of FTC, indicating that the denitrification rate was relatively stable. Interestingly, the NO_3^--N concentration of the whole soil (WH-FT) increased in the first two FTCs and then decreased rapidly in the later period with a larger slop, indicating that the denitrification rate was larger in the following period. This suggests that the inhibition of nitrification was incomplete in these soils.

3.2. Effect of FTCs on soil respiration and N transformations in soil with/without aggregates and different aggregate size classes

3.2.1. Soil respiration

In this experiment, the presence (whole soil) or absence (crushed soil) of soil structure were compared to soil from different aggregate size fractions for soil respiration under freezing and thawing conditions. The results show that soil respiration was not affect by the presence/absence of soil aggregate, and the freezing and thawing did affect the soil respiration.

In terms of the presence/absence of soil aggregate, the soil respiration of both whole soil and crushed soil that have undergone freezing and thawing cycles were significantly lower than that of soils held at 5℃ (Fig.9), indicating that the freezing and thawing inhibited soil respiration. The possible explanations include, 1) the suppression of soil respiration of the FT group during the freezing period at -5 ℃, or 2) the short melting period of 3 days is not long enough to allow soil microbial activity to consume the organic substrates released as a result of freezing. The soils held at 5 ℃ provided a greater amount of heat units to support microbial metabolism, a total of 150 degree-days above zero (30 days * 5 ℃) compared to only 75 degree-days in the FTC treatments (15 days * 5 ℃). Despite the lower thermal regime, soil respiration was only 10% less in the whole and crushed soils and was not different in the aggregate size fractions. Many previous studies have suggested that the increase in substrate concentration in soil is the release of protected

organic matter by damaging the structure of soil aggregates, which stimulates microbial activity and resulting in increased soil respiration. Often, these studies only consider respiration on thawing period and are conducted at warmer thawing conditions (Henry, 2007). However, from the results of this study, the CO_2 accumulation of crushed soil remained at 5 ℃ was not significantly different from whole soil held at 5 ℃, and the CO_2 gathering were 883 and 890 mg·kg$_{-1}$·soil, respectively. A release of soluble organic C may have partially offset the reduced time period over which the microbial community would have been active. The crushing treatment did not affect soil respiration, suggesting that microbial metabolism was more limited by environmental condition (temperature) than substrate availability.

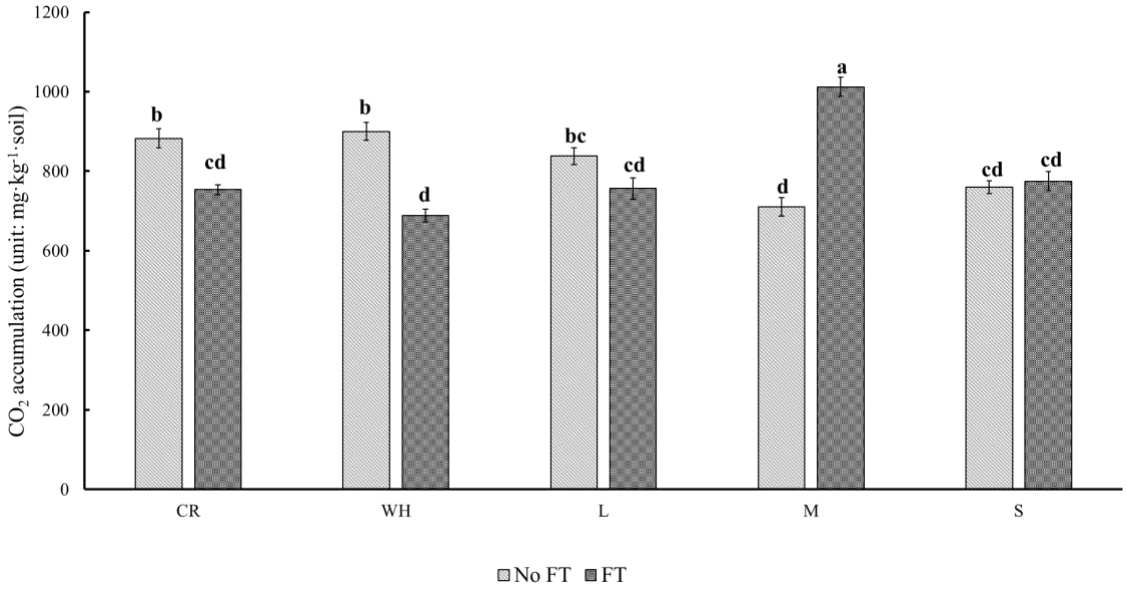

Figure 9: Cumulative CO_2 of total of five FTCs on study 2 (unit: mg·kg$_{-1}$·soil), where CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; L means the large size aggregate fraction (4 to 8 mm); M means the medium size aggregate fraction (0.25 to 4 mm); S means the small size aggregate fraction (0 to 0.25 mm); No FT means the treatment that maintained at 5 ℃ environment; FT means the

treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

As for different aggregate size fractions (Fig.9), in the soil maintained at 5 ℃, the CO_2 accumulation of the largest aggregate size fraction was 838 mg·kg^{-1}·soil, which was significantly higher than that of medium (710 mg·kg^{-1}·soil), indicating that larger aggregates were barely higher soil respiration. This means that soil microorganisms can more easily obtain more substrates from large aggregates. The study by Monreal et al. (1997) have shown that as the size of aggregates increases, the oxidative stability of soil organic matter gradually decreases, and the turnover time of soil organic matter became longer as the size of aggregates decrease due to the association between organic and mineral constituents in microaggregate.

After freeze-thaw treatment, soil respiration of large and small aggregates did not change significantly. However, the respiration of medium-sized aggregates increased considerably from 710 to 1012 mg·kg^{-1}·soil, suggesting that the freeze-thaw treatment promoted the release and subsequent respiration of carbon substrates contained in the medium-sized aggregates. The reason for this phenomenon may be that there is a greater percentage of particulate soil organic matter, generally classified in the 0.25 to 2mm size fraction (Cambardella & Elliott, 1992; Gregorich et al., 2008), which is also the size range of the medium-sized aggregates in this study. Freezing and thawing may cause the organic matter in the soil particles to expand and contract under the action of temperature which causes its internal hydrogen bonds to break and released smaller organic molecules, making it easier to be available by soil microorganisms (Larsen et al., 2002). The concentration of

dissolved organic carbon needs to be further explored for proving this speculation.

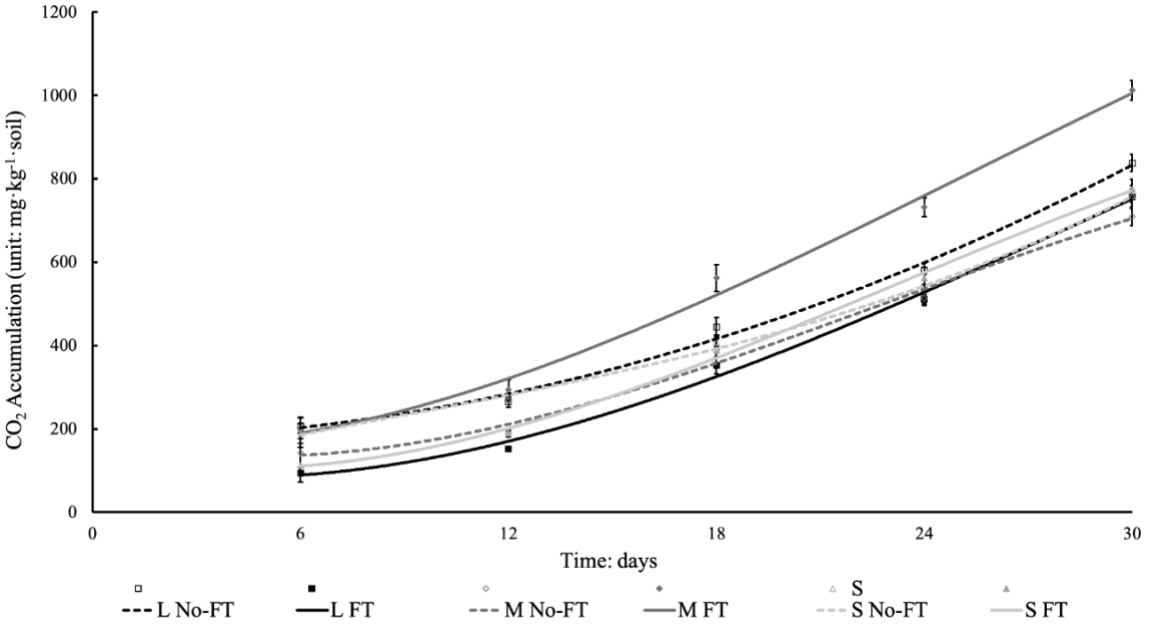

Figure 10: Curves of CO_2 accumulation in different soils aggregate size over time (unit: mg·kg^{-1}·soil); where FTC means the number of freezing and thawing cycles; CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; L means the large size aggregate fraction; M means the medium size aggregate fraction; S means the small size aggregate fraction; No FT means the treatment that maintained at 5 ℃ environment; FT means the treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

Carbon dioxide accumulation in different soil aggregate sizes over time are shown in Fig.10. In general, the respiration rates of each aggregate were relatively slow in the early stage due to the temperature change of the incubated environment from 15 ℃ to -5 / 5 ℃. Compared with the soil held at 5 ℃, the CO_2 production increased more rapidly in aggregate fractions subjected to freezing and thawing, indicating that the freeze-thaw treatment accelerated the respiration rate of each aggregate fraction, especially in the medium size aggregate.

3.2.2. Nitrogen mineralization

This study was conducted to examine the effect of freezing and thawing on the N mineralization of soil as influenced by soil structure and aggregate size fractions at 60% WFPS. ANOVA documented a significant effect of freezing and thawing on the N mineralization of soil and that this effect was influenced by soil structure and aggregate size (Table 2). At a soil moisture content of 60% WFPS, there was a significant interaction between soil structure and FTC in influencing N mineralization. However, there was no difference in the N mineralization in the aggregate size fractions, exposure to FTCs, or their interaction.

Table 2. The effect of freezing and thawing on the amount of N mineralized of soil as affected by different soil structures and aggregate size (unit: mg·kg^{-1}·soil). Means followed by different letters indicate significant soil structure X incubation interaction effects.

	Soil structure		Aggregate size fraction		
	Whole	Crushed	Large	Medium	Small
No FT	1.79±0.31 ^{b}	3.29±0.92 ^{ab}	3.48±0.37	2.60±0.15	2.05±0.74
FT	3.75±0.06 ^{a}	2.56±0.63 ^{ab}	3.35±0.78	4.18±0.28	2.72±0.60
Soil structure		n. s.	Aggregate size		n. s.
Incubation		n. s.	Incubation		n. s.
Soil structure X incubation		*	Aggregate size X Incubation		n. s.

Mean values ± standard errors are shown;
* significant at 5% level;
n. s. no significant difference

The effect of freezing and thawing on the N mineralization of soil, as affected by different soil structures, can be seen from (Fig.11). As a result of repeated FTCs, the N mineralization of the whole soil doubled, from 1.79 to 3.75 mg·kg^{-1}·soil, but the same situation did not occur when the soil aggregates were crushed prior to freeze thaw treatment.

Freezing and thawing did not significantly impact the N mineralization of crushed soil which was 3.29 mg·kg-1·soil in No-FT treatment and 2.56 mg·kg-1·soil in FT treatment. In other words, the increased N mineralization observed in the whole soil with successive FTCs was dependent on maintaining the aggregate structure of the soil. The same results were also observed in another study of this project (Section 3.1.2). This may indicate that crushing alters the structural features that result in changes of substrate availability for microorganisms in the soil during freezing and thawing.

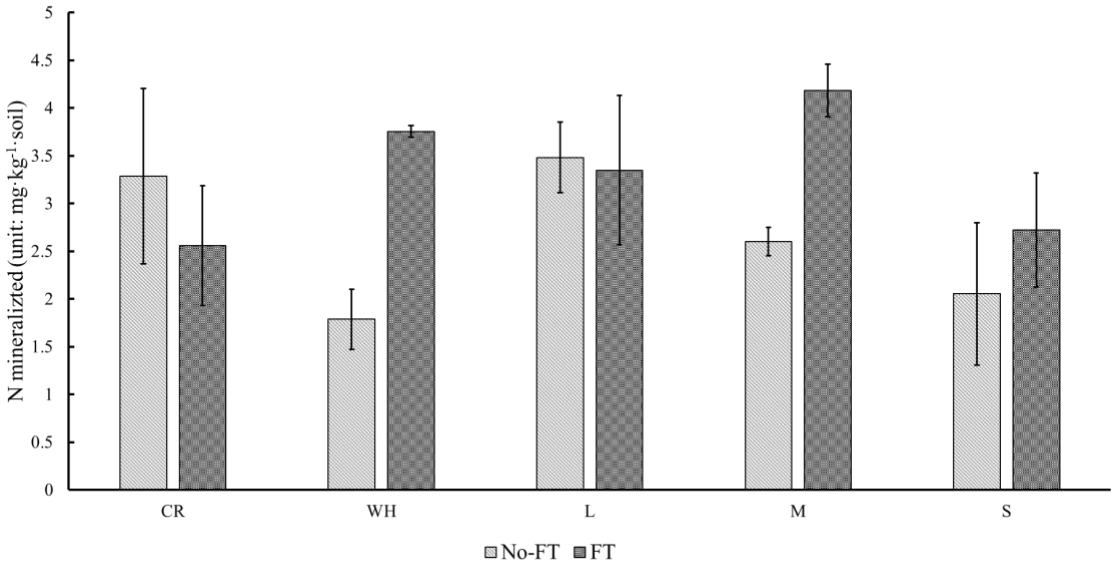

Figure 11: Amount of N mineralization of total of five FTCs on study 2 (unit: mg·kg-1·soil), where CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; L means the large size aggregate fraction; M means the medium size aggregate fraction; S means the small size aggregate fraction; No FT means the treatment that maintained at 5 ℃ environment; FT means the treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

There was a week relationship between N mineralization and soil respiration (R_2 = 0.14) which was not impacted by freezing and thawing (Fig. 12). In both FT and no FTC

treatments there was a week positive relationship between the rate of respiration and N mineralization, indicating the N mineralization increases with increasing soil respiration (Fig. 12). Since soil respiration represents soil microbial activity, higher soil microbial activity promoted the N mineralization. Especially, at the medium size aggregates, the presence of particular organic matter enhanced the microorganism activity after FTCs, which also promoted the N mineralization.

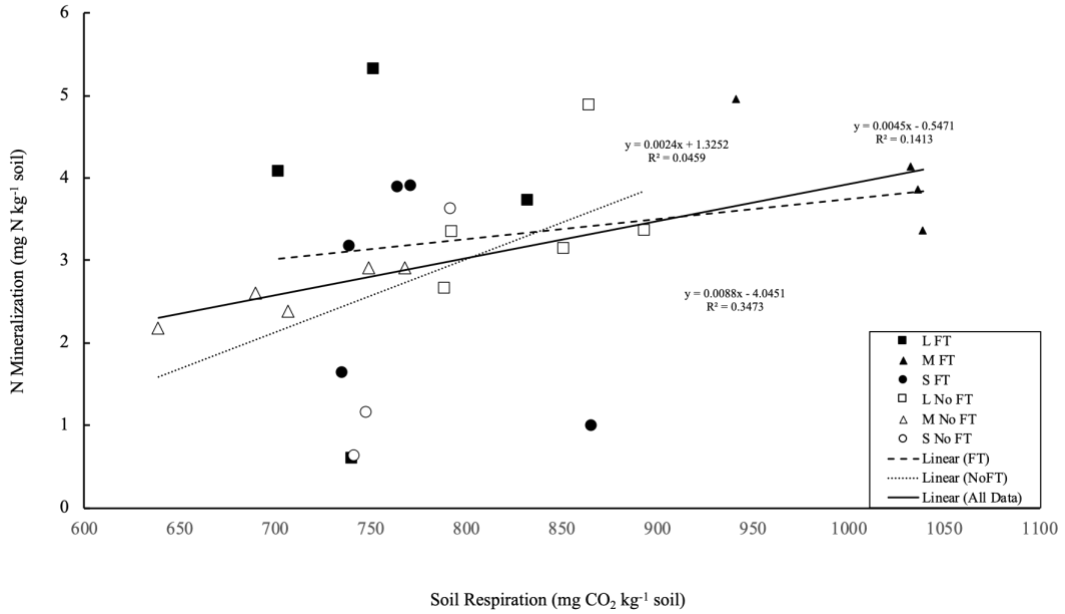

Figure 12: The scatterplot of soil respiration vs. N mineralization of soil aggregate size fractions (S, M, L) undergoing freeze thaw cycles (FT) or no freeze-thaw cycles (No FT).

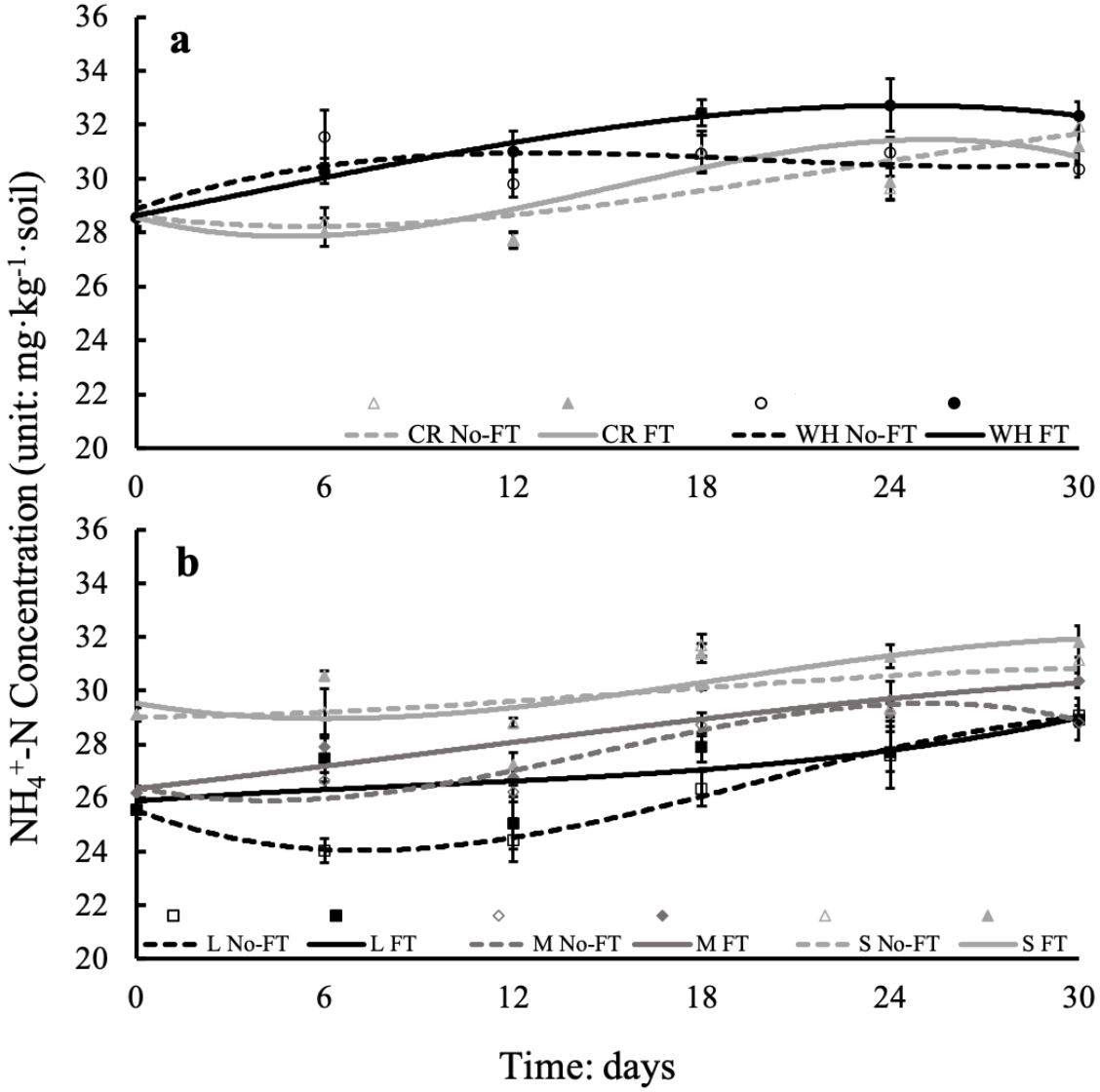

Figure 13: a) Curves of NH_4^+-N concentration in different soils structure over time; b) Curves of NH_4^+-N concentration in different soils aggregate size over time (unit: mg·kg^{-1}·soil); where FTC means the number of freezing and thawing cycles; CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; L means the large size aggregate fraction; M means the medium size aggregate fraction; S means the small size aggregate fraction; No FT means the treatment that maintained at 5 °C environment; FT means the treatment that experienced successive freezing and thawing cycles.

The NH_4^+-N concentration of whole soil that were exposed to successive FTCs (WH-FT) increased rapidly in the early phase, and then flattened (Fig.13a). It may be that a large

amount of organic nitrogen was released rapidly in the first few freezing-thawing cycles, and as the number of FTCs increased, the amount of substrate released decreases, leading to a decrease in the release amount of NH_4^+ after freezing-thawing. Also, The NH_4^+-N concentration of whole soil held at 5 ℃ (WH-No FT) rose slowly after unexplained fluctuations in the first 6 days. As a comparison with whole soil, the upward trend of NH_4^+-N concentration in soils where the aggregates were crushed was more variable. This was especially true for the crushed soil treated subjected to freezing and thawing (CR-FT). On the other hand, although no significant difference in N mineralization was observed among the three different aggregate size fractions, some information can still be found from the curves of NH_4^+-N concentration in different aggregate size fractions over time (Fig.13b). In general, for all three aggregate size fractions subjected to FTCs, the increase of the NH_4^+-N concentration was slow but consistent. However, for the soil held at 5 ℃, large fluctuations in NH_4^+-N concentrations was observed implied the incomplete inhibition of nitrification.

3.2.3. Nitrate and Denitrification

This study investigated the effects of soil structure and aggregate size distribution on soil NO_3-N concentration and denitrification as influenced by freezing and thawing. Soil structure was an essential factor affecting denitrification and freezing and thawing of soil aggregates of different size classes stimulated denitrification (Fig.14). The soil structure and FTC treatment interacted significantly in influencing denitrification. In terms of soil structure for the soils incubated at constant temperature (No FT, Fig.14), crushing soil aggregates significantly reduced denitrification from 2.21 to 0.57 mg·kg-1·soil·, suggesting crushing inhibited conditions conducive to denitrification. The freeze-thaw treatment did not significantly affect the denitrification of the whole soil, but significantly increased the denitrification of crushed soil to 1.68 mg·kg-1·soil·, which was the same level of the whole soil's denitrification. A possible explanation is that, when the water content was sufficient, there was a layer of water film on the surface of the soil aggregates that was adsorbed on the surface by means of molecular adsorption (Leão & Tuller, 2014). This layer of water film and soil aggregates form a closed space. When the oxygen in the closed space was consumed entirely, a micro-environment suitable for denitrification was created. Once the soil aggregate structure was destroyed the aeration condition of the soil was improved, so there was less anaerobic space in the soil. Denitrification was inhibited due to sufficient oxygen availability. When the soil was frozen, due to the generation of ice crystals, the soil pores were enlarged due to water expansion, and the ice film was generated (Teepe et al., 2001), hence, the closed space where denitrification can occur was re-created.

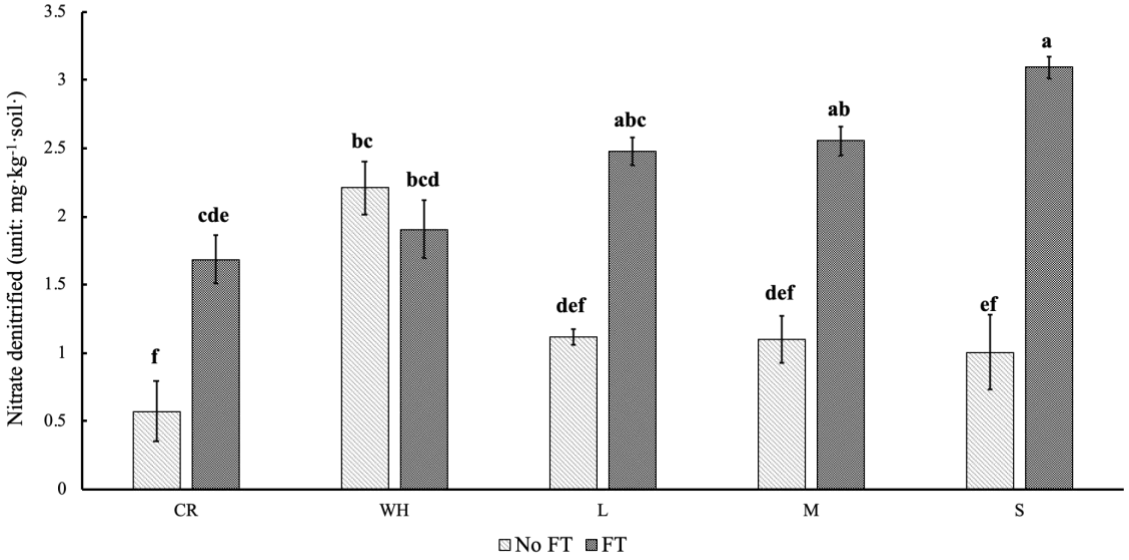

Figure 14: The amount of nitrate denitrified on study 2 (unit: mg·kg-1·soil·), where CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; L means the large size aggregate fraction; M means the medium size aggregate fraction; S means the small size aggregate fraction; No FT means the treatment that maintained at 5 ℃ environment; FT means the treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

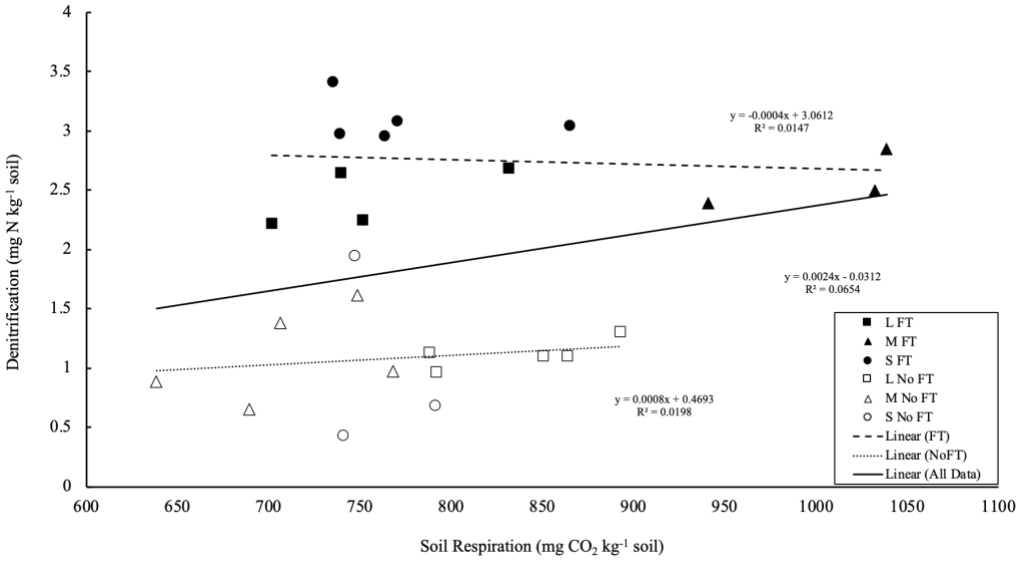

Figure 15: The scatterplot of soil respiration vs. denitrification of soil aggregate size fractions (S, M, L) undergoing freeze thaw cycles (FT) or no freeze-thaw cycles (No FT).

As for the soil aggregate size fractions (Fig.14), there was no significant difference among each size fraction. However, all three size fractions had a significant increase in denitrification in response to FTCs. The amount of denitrified nitrate increased by an average of 1.63 mg·kg^{-1}·soil·, which is about 2.5 times of the original. Interestingly, the whole soil was not significantly affected by freezing and thawing. The response of each aggregate fractions on the denitrification by freeze-thaw were consistent with that of crushed soil, which implies that a certain amount of aggregates was destroyed during the sieving and compressing processes. When examining the entire data set, denitrification demonstrated a week tendency to increase with increasing respiration (R_2 = 0.07), but separation of soils subject to FTCs from those maintained at 5 C it was apparent that there was no relationship with the magnitude of respiration (Fig. 15). As noted earlier, higher rates of denitrification occurred in the soils subject to FTCs independent of soil respiration. This suggests that the magnitude of denitrification was not a function of the magnitude of microbial activity but was enhanced in soils subject to FTCs by some other mechanism.

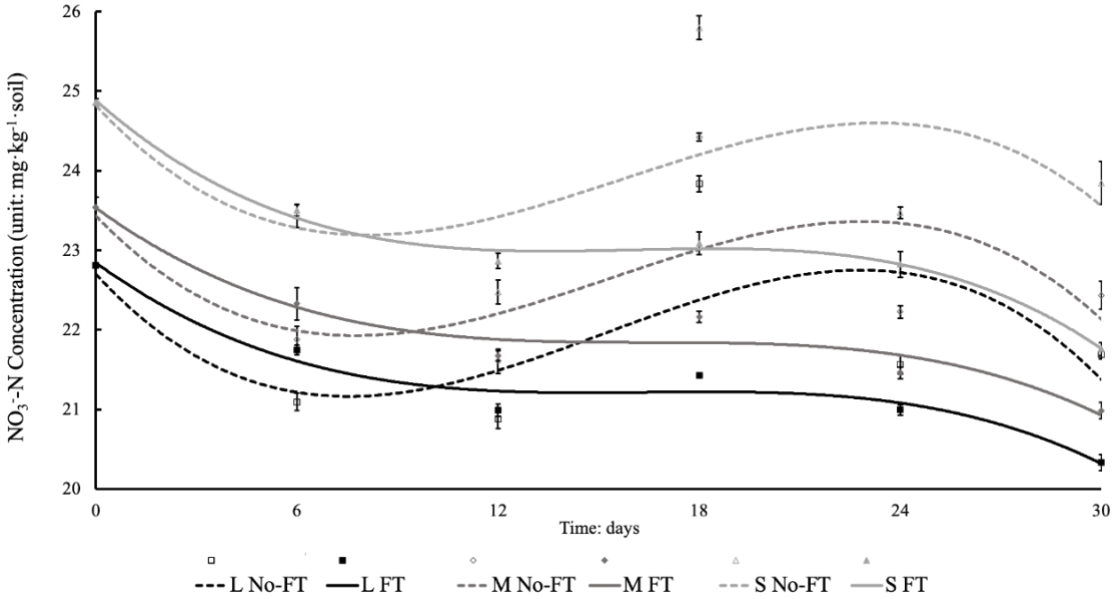

Figure 16: Curves of NO_3-N concentration in different soils aggregate size and FTC treatment over time (unit: mg·kg^{-1}·soil); where FTC means the number of freezing and thawing cycles; CR means the soil treatment that the aggregates were removed; WH means the soil remained the aggregates; L means the large size aggregate fraction; M means the medium size aggregate fraction; S means the small size aggregate fraction; No FT means the treatment that maintained at 5 ℃ environment; FT means the treatment that experienced successive freezing and thawing cycles. Mean value and standard error of the mean are presented.

In general, regardless of whether the soil has undergone freeze-thaw treatment, the concentration of NO_3-N had the same trend, indicating that with the decreased of the aggregates size, the concentration of NO_3-N raised (S > M > L) (Fig.16). Because the concentration of NO_3^- ions added in the FT0 stage were the same, it means that the difference in NO_3-N concentration between different size fractions is mainly attributed to the aggregates themselves. For the same mass of soil, the smaller the size of the soil aggregate means the larger expose of specific surface area. Therefore, the more NO_3^- ions that can be adsorbed, resulting in the higher extracted NO_3-N concentration (Black & Waring, 1979). From Figure 16, after the freeze-thaw treatment (FT), the NO_3-N

concentration of the aggregates at all levels decreased more steadily with the increase in the number of freeze-thaw cycles, which means that the denitrification rate of the aggregates at various levels after freeze-thaw treatment was relatively stable. In contrast, the NO_3^--N concentration of the soils held at 5 ℃ decreased rapidly in the early stage, especially in FTC1 (the denitrification rate was even higher than the aggregates that had been subjected to freeze-thaw treatment). Subsequently, the NO_3^--N concentration increased, suggesting incomplete inhibition of nitrification. A fluctuation occurred in the third FTC might cause by failure of nitrification inhibitor (Zebarth et al., 2019).

Chapter 4. Conclusions

It can be concluded that successive freeze-thaw cycles greatly affect the respiration and nitrogen dynamics on the soil, and the roles of soil moisture content and soil structure as critical influencing factors cannot be ignored.

Freeze thaw cycles inhibited soil respiration, but the respiration was enhanced at the thawing period. However, removed the soil structure directly did not affect soil respiration, which implies the protected substrates by aggregate structure was not the main factor to result in the burst of CO_2 in this project. Freezing and thawing promoted N mineralization but crushing of soil aggregates eliminated the effect of FTCs, which indicates that crushing alters the structural characteristics and results in changes in the mechanisms of solubilization of substrates and/or substrate utilization by microorganisms in the soil during freezing and thawing. In addition, freezing and thawing did not affect the denitrification of the whole soil but significantly increased the denitrification in the crushed soil. Particularly, at 60% WFPS, the interaction between crushing and freezing and thawing enhanced denitrification to a level equivalent to or greater than that of whole soil. Further, the freeze-thaw effect has a more significant impact on medium size aggregates (0.25 to 4mm). Medium-sized aggregates respond strongly to the freeze-thaw impacts in soil respiration and N mineralization. Freezing and thawing enhanced denitrification of aggregates on all three size fractions.

Unfortunately, due to the Cox fire, the assessment of soil carbon and nitrogen pools

(total carbon and total nitrogen) that should have been included in this project could not be completed. Also, this study contains a series of reasonable assumptions based on soil-water dynamics, but there is no direct evidence to prove this study more directly. We hope that this research will enable scholars in this field to explore further.

This project investigated and discussed the effects of freeze-thaw on soil carbon and nitrogen and their possible mechanism, which will help to further determine the characteristics of soil carbon/nitrogen changes and their effects on plant growth before and after freeze-thaw. It is of great significance to regulate fertilization, increase N use efficiency, reduce soil greenhouse gases emission, and promote sustainable development of soil ecosystems.

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Appendices

Table A.1: Analysis of variance (ANOVA) of CO_2 Accumulation of total of five FTCs with factors of soil treatments, water contents (WFPS), incubation conditions, and their interactions on Study 1 (unit: mg·kg^{-1}·soil). The response was translated by negative square root (^-0.5) for passing normality test.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Aggregate	1	0.000004	0.000004	7.59	0.009
WFPS	2	0.000031	0.000015	26.30	0.000
Incubation	1	0.000045	0.000045	77.85	0.000
Aggregate X WFPS	2	0.000009	0.000004	7.37	0.002
Soil X Incubation	1	0.000078	0.000078	133.44	0.000
WFPS X Incubation	2	0.000023	0.000011	19.71	0.000
Soil X WFPS X Incubation	2	0.000014	0.000007	12.12	0.000 *
Error	40	0.000023	0.000001		
Total	51	0.000229			

* Significant difference

Table A.2: Analysis of variance (ANOVA) of the amount of N mineralized after five FTCs with factors of soil treatments, water contents (WFPS), incubation conditions, and their interactions on Study 1 (unit: mg·kg^{-1}·soil).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Aggregate	1	11.102	11.102	8.71	0.005
WFPS	2	73.759	36.879	28.92	0
Incubation	1	7.581	7.581	5.95	0.019
Aggregate X WFPS	2	22.233	11.116	8.72	0.001 *
Soil X Incubation	1	16.145	16.145	12.66	0.001 *
WFPS X Incubation	2	5.232	2.616	2.05	0.14
Soil X WFPS X Incubation	2	5.989	2.994	2.35	0.106
Error	48	61.21	1.275		
Total	59	203.251			

* Significant difference

Table A.3: Analysis of variance (ANOVA) of the amount of nitrate denitrified after five FTCs with factors of soil treatments, water contents (WFPS), incubation conditions, and their interactions on Study 1 (unit: mg·kg$_{-1}$·soil).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Aggregate	1	0.0189	0.01891	0.17	0.678
WFPS	2	3.4085	1.70424	15.75	0
Incubation	1	5.8961	5.89609	54.49	0
Aggregate X WFPS	2	4.2356	2.11778	19.57	0
Soil X Incubation	1	2.8812	2.88124	26.63	0
WFPS X Incubation	2	1.0754	0.5377	4.97	0.011
Soil X WFPS X Incubation	2	1.0578	0.52888	4.89	0.012 *
Error	43	4.6526	0.1082		
Total	54	22.3419			

* Significant difference

Table A.4: Analysis of variance (ANOVA) of CO_2 Accumulation of total of five FTCs with factors of soil treatments, incubation conditions, and their interaction on Study 2 (unit: mg·kg$_{-1}$·soil).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Soil	4	40767	10192	5.03	0.003
Incubation	1	4569	4569	2.25	0.143
Soil X Incubation	4	337481	84370	41.63	0.000 *
Error	32	64853	2027		
Total	41	431407			

* Significant difference

Table A.5: Analysis of variance (ANOVA) of the amount of N mineralized after five FTCs with factors of soil structure, incubation conditions, and their interaction on Study 2 (unit: mg·kg$_{-1}$·soil).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Soil Structure	1	0.114	0.114	0.07	0.797
Incubation	1	1.9158	1.9158	1.15	0.3
Soil Structure X Incubation	1	9.0828	9.0828	5.44	0.033 *
Error	16	26.7191	1.6699		
Total	19	37.8317			

* Significant difference

Table A.6: Analysis of variance (ANOVA) of the amount of N mineralized after five FTCs with factors of aggregate size fractions, incubation conditions, and their interaction on Study 2 (unit: mg·kg^{-1}·soil).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Aggregate size	2	6.897	3.448	2.35	0.117
Incubation	1	3.741	3.741	2.55	0.123
Aggregate size X Incubation	2	3.688	1.844	1.26	0.302
Error	24	35.158	1.465		
Total	29	49.483			

Table A.7: Analysis of variance (ANOVA) of the amount of nitrate denitrified after five FTCs with factors of soil treatments, incubation conditions, and their interaction on Study 2 (unit: mg·kg^{-1}·soil).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Soil	4	5.318	1.3294	9.22	0
Incubation	1	15.502	15.5024	107.57	0
Soil X Incubation	4	7.812	1.9531	13.55	0 *
Error	38	5.476	0.1441		
Total	47	33.328			

* Significant difference