TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Bodenökologie

Effects of freezing and thawing on nitrification and denitrification in arable soil

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzende: Univ.-Prof. Dr. Jean Charles Munch

Prüfer der Dissertation:

1. Hon.-Prof. Dr. Michael Schloter
2. Univ.-Prof. Dr. Johannes Kollmann

Die Dissertation wurde am 27.08.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 18.10.2012 angenommen

一份耕耘，一份收获 —— 谚语

（No pain, no gain）

致我的父亲母亲……

For my parents…

Contents

List of publications and contributions

Publications

Ⅰ. Su, M.X., Kleineidam, K., Schloter, M. Influence of different litter quality on the abundance of genes involved in nitrification and denitrification after freezing and thawing of an arable soil; Biology Fertility of Soils 46, 537 - 541 (2010)

Ⅱ. Ollivier, J., Töwe, S., Bannert, A., Hai, B., Kastl, E., Meyer, A., Su, M.X., Kleineidam, K., Schloter, M. Nitrogen turnover in soil and global change; FEMS Microbiol Ecol (2011) 1–14

My contributions to the publications

Ⅰ. I took part in planning and carrying out all the experiments and the following statistic analyses. The manuscript is based mainly on my input.

Ⅱ. I contributed to the manuscripts writing about the freeze-thaw influence in the section “Changing climatic conditions”.

Abstract

Due to increased awareness of climate change during the last few decades, more researchers have focused on the influence of freezing and thawing on the nitrogen (N) cycle processes in soil. It has long been accepted that freezing and thawing cycles disrupt soil aggregates and lead to cell lysis, releasing organic carbon (C) and nitrogen into the soil, and increasing microbial N transformation processes.

In this study 3 microcosm experiments were performed to investigate the influence of different types of plant litter on nitrification and denitrification patterns in the context of freezing and thawing in the soil. In addition, the influence of copper on these patterns was studied.

In the first microcosm study, the influences of legume-grass mixtures and wheat straw residues with different C/N ratios were investigated on N transformations, while noting the abundance patterns of the corresponding functional genes in an arable soil after freezing and thawing. Soil samples were amended with similar C quantities of the two plant residues resulting in different N contents. Unfrozen soil samples, continuously incubated at 10 °C, served as controls. Samples were taken before freezing and at 1, 3 and 7 days after thawing. The concentration of the soil chemicals, as well as the *amoA* genes involved in ammonia oxidizers, nitrite and the N_2O reducers *nirK*, *nirS* and *nosZ* were determined. The amounts of inorganic N in the forms of TNb, NH_4^+ and NO_3^- and WEOC as well as the investigated gene abundance patterns hardly differed between the control sample, and freezing and thawing treated samples which amended with wheat straw. In contrast, clear differences were observed after freezing and thawing in samples using the legume-grass mixtures as the amendment compared to the controls.

The second microcosm study was similar to the first experiment; except that similar N quantities of plant residues were used, resulting in different C amounts added to the soil samples. Significant differences in the amounts of inorganic N in the forms of TNb and NH_4^+ as well as the *amoA* AOB gene abundances were detected between the freeze-thaw treated and non-frozen control samples that had used the legume-grass mixtures. On the other hand, clear alterations of *amoA* AOB and *nosZ* gene copy numbers were observed in wheat straw samples after freezing and thawing compared to the control samples. Significantly, the impact of different plant residues was more pronounced than the influence of freezing and thawing.

In the third experiment, the influence of Cu^{2+} was investigated. Microcosms were amended with legume-grass mixtures and treated with or without Cu^{2+}. In addition to this analysis, the diversity of microbes in nitrification and denitrification was detected by T-RFLP. Cu^{2+} contamination had strong effects, not only on the organic C and N contents of the soil, but also on the abundance of genes involved nitrification and denitrification and the corresponding N transformations during freezing and thawing events. Analysis of microbial communities concluded that Cu^{2+} contamination enhanced the freeze-thaw influence on microbial diversity, whereas, freeze-thaw stress weakened the Cu^{2+} contamination effects.

Altogether, the series of laboratory incubation experiments indicated that the abundance of ammonia oxidizers and nitrite reducers in soil was negatively correlated with additional C input by residues. Surprisingly, the impact of plant residues was more pronounced than that of freeze-thaw stress.

Zusammenfassung

Auf Grund des voranschreitenden Klimawandels während der letzten Jahrzehnte, ist der Einfluss von Frost-Tau-Zyklen auf den Stickstoffkreislauf in Böden immer mehr in den Fokus der Wissenschaft gerückt. Es ist bekannt, dass Frost-Tau Ereignisse Bodenaggregate zerstören und die Zelllyse erhöhen, was zu vermehrter Freisetzung organischer Kohlenstoff und Stickstoffverbindungen und zur Steigerung mikrobieller Stickstofftransformationsprozesse führen kann.

Im Rahmen dieser Doktorarbeit wurden 3 Mikrokosmenversuche durchgeführt, um den Einfluss von verschiedenen Pflanzenmaterialien und der Applikation von Kupfer auf die Nitrifikation und Denitrifikation nach einem Frost-Tau Ereignis zu untersuchen. Im ersten Versuch wurde der Einfluss von Pflanzenmaterial mit unterschiedlichem C/N Verhältnis (Leguminosen-Gras Mischung und Weizenstreu) in einem Ackerboden auf die Abundanz der funktionellen Gene des Stickstoffkreislaufs nach einem Frost-Tau Ereignis betrachtet. Die Pflanzenstreu wurde so eingesetzt, dass gleiche Kohlenstoffmengen appliziert wurden, was wiederum zu unterschiedlichen Stickstoffmengen führte. Zur Kontrolle wurden Proben kontinuierlich bei 10°C inkubiert. Proben wurden vor dem Einfrieren und 1, 3 und 7 Tage nach dem Frost-Tau Ereignis genommen. Es wurden sowohl bodenchemische Parameter (WEOC, TNb, NH_4^+ and NO_3^-) als auch die Abundanz der funktionellen Gene, die bei der Nitrifikation (*amoA* AOA, AOB) und Denitrifikation (*nirK, nirS, nosZ*) eine Rolle spielen, bestimmt. Die Proben, die mit der Weizenstreu behandelt wurden, zeigten nur geringe Unterschiede in der Menge des anorganischen Stickstoffes (TNb, NH_4^+ and NO_3^-), WEOC und den Genabundanzen zwischen der Kontrolle und den Frost-Tau behandelten Proben. Im Gegensatz dazu, führte das Frost-Tau Ereignis zu eindeutigen Unterschieden bei den bodenchemischen Parametern und Genabundanzen der Proben, die mit der Leguminosen-Gras Mischung behandelt wurden.

Im zweiten Versuch wurden gleiche Mengen Stickstoff auf den Ackerboden gegeben, was wiederum zu einem unterschiedlichen Kohlenstoffeintrag führte. Ansonsten war die Durchführung mit dem ersten Versuch vergleichbar. Es wurden signifikante Unterschiede im anorganischen Stickstoffgehalt (TNb und NH_4^+) als auch der *amoA* AOB Abundanz zwischen Kontrollen und Frost-Tau behandelten Proben gefunden, wenn der Boden mit der Leguminosen-Gras Mischung inkubiert wurde. Die Inkubation mit Weizenstreu dagegen führte zu Unterschieden in der *nosZ* und *amoA* AOB Abundanz, wenn man die Kontrollen und die Frost-Tau behandelten Proben vergleicht. Insgesamt war der Einfluss des Pflanzenmaterials größer, als der Frost-Tau Effekt.

Im dritten Versuch wurde der Einfluss von Cu^{2+} untersucht. Zu diesem Zweck wurden die Bodensäulen zunächst mit der Leguminosen-Gras Mischung versetzt und anschließend mit oder ohne Cu^{2+} inkubiert. Zusätzlich zu den bisherigen Analysen wurde die Diversität der nitrifizierenden und denitrifizierenden Mikroorganismen mittels T-RFLP bestimmt. Bei den Proben, die dem Frost-Tau Ereignis ausgesetzt waren, hat sich ein starker Einfluss der Cu^{2+}-Kontamination auf den organischen Kohlenstoff- und Stickstoffgehalt im Boden, sowie auf die Abundanz von funktionellen Genen der Nitrifikation und Denitrifikation gezeigt. Die Untersuchung der mikrobiellen Diversität ergab, dass die Cu^{2+}-Kontamination den Frost-Tau Effekt erhöht hat, und gleichzeitig das Frost-Tau Ereignis den Einfluss von Cu^{2+} reduzierte.

Abschließend kann man sagen, dass die verschiedenen Mikrokosmenversuche gezeigt haben, dass die Abundanz von Ammoniak-oxidierenden und Nitrit-reduzierenden Mikroorganismen negativ mit dem zugegebenen Kohlenstoff korreliert. Überraschenderweise hat sich gezeigt, dass der Einfluss des Pflanzenmaterials größer ist als der Einfluss des Frost-Tau Ereignisses.

1 Introduction

1.1 Global change

Since the Industrial Revolution started in the 19th Century, the development of industry has accelerated and the World's population has increased over six-fold (Maddison 2003). Such increasing human population has led to global concerns about resource availability (Robert and Lucas 2002). Hand in hand with the development of the Industrial Revolution, came improvements in agricultural technology. This increased soil utilization and crop yields to satisfy increased human demands. However, the consequent problems of pollution, energy shortages and greenhouse gas emissions became more serious. These have caused climate change, widespread species extinctions, desertification, and other large-scale shifts in the terrestrial ecosystems, which are summarised under the term 'Global change' (Carpenter *et al.* 1992, Mosier 1998, Steffen *et al.* 2004, Swift *et al.* 1998, Vitousek *et al.* 1997).

As Fig. 1 shows, global change has altered the structures and functions of ecosystems. These changes, in turn, have affected the availability of ecological resources and the interaction between ecosystems and human activities, including agricultural ecosystems (Gregorich *et al.* 2005, Koponen *et al.* 2004, Skiba and Smith 2000). Therefore there is not only an increasing demand for food production due to the growing world population, but this is also in concert with negative impacts of global change affecting agricultural management all over the world. In certain regions, there have been an increased number of extreme weather events like prolonged periods of drought or heavy rainfall or increased temperature in winter. The latter especially may enhance soil freeze-thaw cycles, resulting in an accelerated loss of the organic fertilizer N from soils. This has been considered as a major concern regarding the sustainable production of plants (Isard and Schaetzl 1998, Kelly and Murphy 2010).

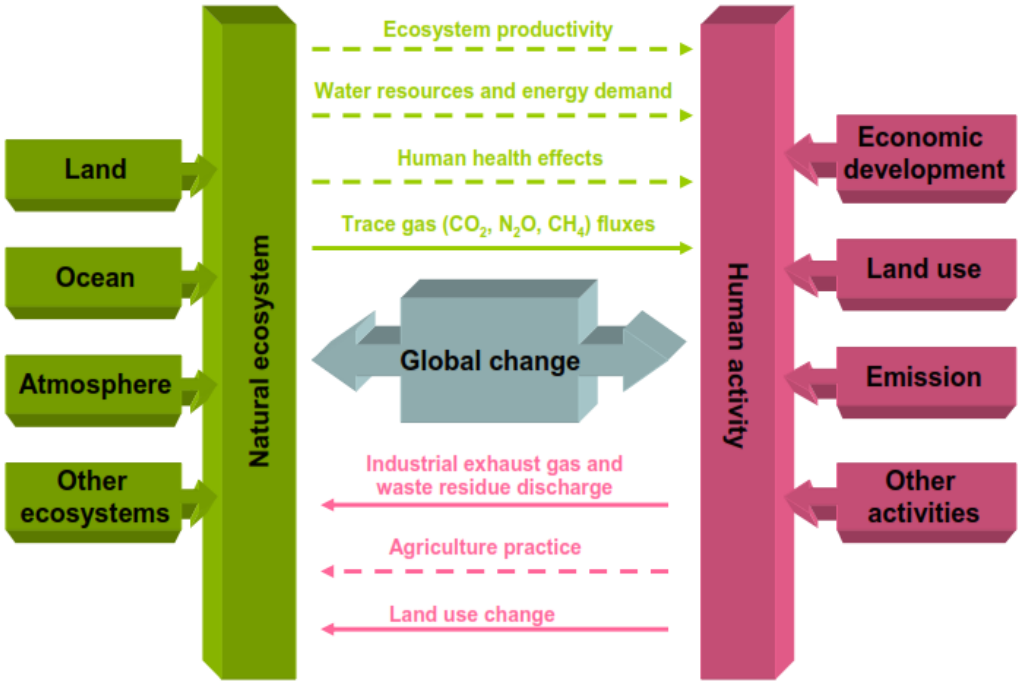

Figure 1 The relationships among natural ecosystems, human activity and global change. The solid arrow lines expressed direct influences and the dotted arrow lines indicated indirect effects.

1.1.1 Climate change

In the past 400,000 years, the climate has shown a periodic cycle of ice ages and warm periods (EEA 2004). However, due to the development of human industrial activities, greenhouse gas emissions have led to global warming. For example, compared with the pre-industrial era before 1750, the atmospheric concentrations of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) have increased by 34%, from 280 to 375 ppm, 153%, from 700 to 1,772 ppb and 17 %, from 270 to 317 ppb, respectively (EEA 2004). Concentrations of CO_2 and N_2O have continued to rise at similar rates in the past decades, at an average of 1.9 ppm per year (EEA 2004, IPCC 2007). Accordingly, the average total global temperature has risen from the 19th to 21st Century by 0.76 °C (IPCC 2007). The average Arctic temperature has increased at a rate nearly twice that of the average global rate in the past hundred years (IPCC 2007). The IPCC (2007) has reported that Arctic temperatures at the top of the permafrost layer have generally increased by up to 3 °C since the 1980s. Since 1900,

the maximum area covered by seasonally-frozen ground in the Northern Hemisphere has decreased by about 7% in wintertime, and by up to 15% in springtime. It has also been suggested that climate change will be accompanied by more extreme climate events like droughts, floods, more very warm days but fewer really cold days and more frequent freeze-thaw cycles (2001b, IPCC 2001a).

In temperate regions in winter, both the air temperature and precipitation rates influence the soil temperature. Global warming can lead to lower soil temperatures and an increase in soil freezing days in wintertime as a result of the reduced snowpack thickness (Brooks and Williams 1999, Isard and Schaetzl 1998). Moreover, in high latitude ecosytems, especially those in permafrost, the length of the snow-covered season is expected to shorten due to global warming (Sparks and Menzel 2002). The number of annual soil freezing days has declined with the increasing mean winter air temperature and precipitation. However, the number of annual soil freeze-thaw cycles has increased in both warm and dry winters. It is predicted that by 2050, the changes in Winter temperatures will have a much stronger effect on the number of annual soil freezing days and freeze-thaw cycles than changes in total precipitation (Henry 2008). Soil freezing-thawing cycles are usually followed by the disruption of soil aggregates, cell lysis, and the subsequent release of nutrients and greenhouse gases, such as N_2O and nitric oxide (NO), as well as CO_2 and CH_4 (Sharma *et al.* 2006). Furthermore, snow removal experiments have revealed that an increased number of soil freezing days and freeze-thaw cycles under a reduced snowpack can accelerate the leaching of C and N (Fitzhugh *et al.* 2001, Groffman *et al.* 2001, Sulkava and Huhta 2003, Weih and Karlsson 2002).

1.1.2 Organic faming as a response of global change

Due to the increasing human population, there is a need for more and better quality food that uses organic fertilizer and less pesticide. Other problems also urgently need to be solved, such as greenhouse gas emissions, soil over used and/or monocultures.

Organic farming relies on advanced techniques such as crop rotation, green manure fertilization, compost and biological pest control (Paull 2011).

Agricultural practices in organic farming include a series of techniques such as fertilisation, tillage, and irrigation. Organic fertilizers are often applied to reduce the amount of N_2O emissions in agricultural soil and to generate well-balanced input and output levels for N. Furthermore, organic fertilizers have been shown to increase the quantity of soil organisms and improve biodiversity and the long-term productivity of soil by providing organic matter and micronutrients (Mäder *et al.* 2002, Prasad *et al.* 2004, Stewart *et al.* 2005). Plant residues in the forms of legume-grass mixtures and wheat straw have been commonly used as 'green manure' in Central Europe during the last few decades (Chèneby *et al.* 2010, Kumar and Goh 1999). These are important sources of C and N for soil microorganisms (Henriksen and Breland 1999, Klemedtsson *et al.* 1991, Tiedje *et al.* 1982). Plant residues increase the percentage of organic biomass in soil, thereby improving the soil's water-holding capacity, soil aeration, and other soil characteristics (Sullivan 2003).

However, there are limitations to the positive effects of plant residues. The incidence of pests and diseases may increase if the residues are not cleared of them. What is more, the plant residues may compete with the crops for soil moisture uptake. However, these effects are strongly dependent upon the quality of the green manure. For example, wheat straw can significantly increase microbial C and N, but can also decrease the amount of soluble organic N in the soil (Zavalloni *et al.* 2011).

Wheat straw leads to higher cumulative CO_2 flux than *Sesbania* which has lower C/N ratio rather compared to legume-grass mixtures. Legume-grasses have a synergetic positive influence on an ecosystem, especially on the N cycle (Li *et al.* 2010). Ledgard and Steele (1992) showed that legume-grass mixtures amended soils showed higher biological N_2 fixation. This minimized the effects of nutrient limitations, reduced soil moisture, lowered soil acidity, and decreased pests and disease.

Furthermore, legume-grass residues can also increase the yield of pasture in grassland (Shehu and Akinola 1995). Lynch *et al.* (2004) confirmed that using legume-grass mixtures may reduce the risk of the excesses or deficits of N content in soils, avoid potential losses to crop yield and quality, and increase the accumulation of soil phosphorus (P) and potassium (K).

The aim of organic farming is not only to improve crop yields but also to reduce the exposure of food to pesticides. Therefore, the use of pesticides, herbicides, insecticides or fungicides, is strongly restricted. Pesticides have a direct, rapid effect on pest and disease control in agricultural management. Heavy metals, including arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), silver (Ag) and zinc (Zn) are important components of pesticides (Nasim *et al.* 2008, Nene and Thapliyal 1979). However, a high heavy metal content can cause harmful effects in agricultural soils through the decomposition of organic matter by soil microorganisms, and on the net fluxes of soil C and N through mineralization and immobilization processes (Nannipieri *et al.* 2003). Heavy metal contamination causes a rapid, negative effect on denitrification activity in soil and sediment. Therefore, denitrification tests can be used to assess the bioavailability of heavy metals in soil (Bardgett *et al.* 1994, Speir *et al.* 1999). Although the use of pesticides is limited in organic farming, Cu^{2+} is still frequently used due to it has less toxicity for plants at low concentrations of no more than 150 - 200 mg total Cu kg^{-1} with 60 - 80 mg bioavailable Cu kg^{-1} (Fernández-Calviño *et al.* 2010).

1.2 Nitrogen cycle processes

N is one of the most indispensable macronutrients for all living beings since it is an essential component of important polymers like amino acids, proteins, and nucleotides (Hooper and Johnson 1999). N enters soil through organic pathways such as plant materials, dead animals and microorganisms. The dynamics of organic N in soil is

reflected by the processes of mineralization, immobilization, leaching and plant uptake (Murphy *et al.* 2000). However, inorganic forms of N, such as NH_4^+, ammonia (NH_3), NO_3^-, nitrite (NO_2^-) can also be directly taken up by plants and microorganisms (Geisseler *et al.* 2010). Inorganic N enters soil mainly through fertilisation, microbial N_2 fixation, converting N_2 into NH_4^+ and the mineralization of complex compounds like proteins.

The N cycle includes several transformative processes performed by microorganisms. As shown in Figure 2, the main processes of terrestrial N cycling are N mineralization, biological N_2 fixation, nitrification and denitrification. Besides these, other processes in the N cycle such as anaerobic ammonium oxidation (Anammox) and dissimilatory nitrate reduction to ammonium (DNRA) mainly occur under nitrate and oxygen limitation (Humbert *et al.* 2010, Mahne and Tiedje 1995, Mohan and Cole 2007, Mulder *et al.* 1995, Rütting *et al.* 2011).

During the processes of the N cycle, N can shift between organic and inorganic forms. For example, NO_3^- and NH_4^+ can be transformed into organic N by plants and other organisms. Organic N can be converted back to inorganic forms by the decomposition of organic compounds. N can also shift among inorganic forms, for example, from NH_3 to NH_4^+ and from NO_3^- to N_2 (Galloway *et al.* 2004, Jenkinson 2001, Maathuis 2009). Which N compounds will actually be assimilated depends upon the atmosphere, soil types, climatic conditions, microorganisms and the plant species (Maathuis 2009).

N can also leave a soil system by leaching or transforming to gaseous forms. N leaching from agricultural soils can represent a substantial loss of fertilizer N which is relevant to microorganism-dependent nitrification and denitrification processes. NO_3^- as the product of nitrification can easily leach; on the other hand, gaseous forms of N such as NO, N_2O and N_2 are produced during the denitrification processes (Olson *et al.* 2009, Riley *et al.* 2001).

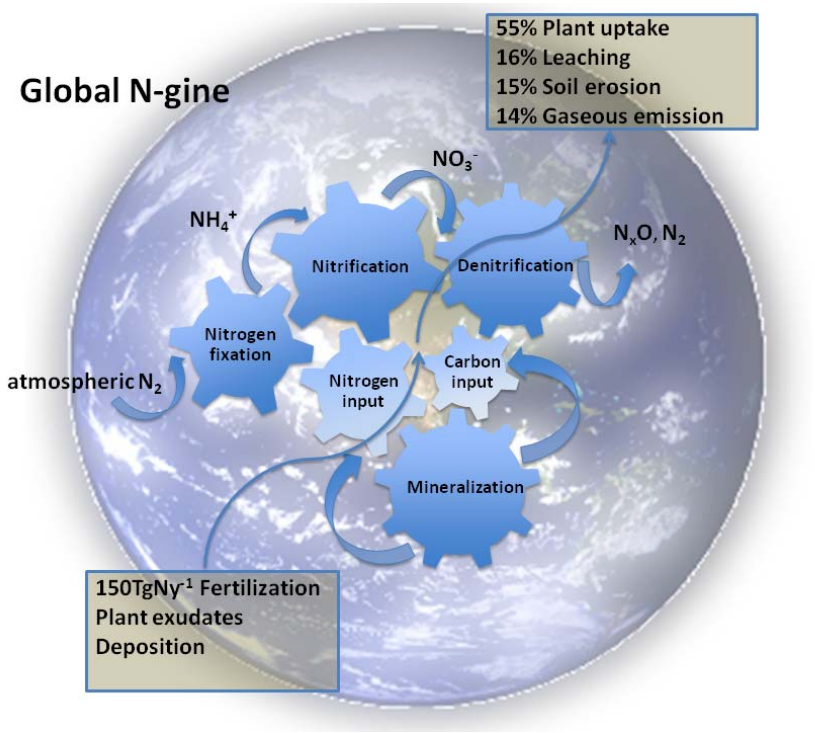

Figure 2 A simplified sketch of the N cycle in soils. It includes N_2 fixation, nitrification, denitrification, mineralization, biomass synthesis. (From Publication II).

Nitrification and denitrification processes are 2 of the most important steps in N cycle. They related to the greenhouse gas emission (N_2O) which significant influenced by freezing and thawing stress (Henry 2007, Matzner and Borken 2008, Skiba and Smith 2000). Due to the damage of global warming effects it is worth to continue the research on nitrification and denitrification to improve our knowledge about how to reduce the N_2O emission under the freeze-thaw stress.

1.2.1 Nitrification

Nitrification is an aerobic process with two enzymatic oxidation steps in oxidizing NH_3 to NO_3^-. This is shown in Figure 3. In the first step, NH_3 is oxidized into NO_2^- by ammonia-oxidizing bacteria (AOB), e.g. *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus* and *Nitrosospira*) or ammonia-oxidizing archaea (AOA, e.g. *Nitrosopumilaceae*) (Boyle-Yarwood *et al.* 2008, Gubry-Rangin *et al.* 2010, Leininger *et al.* 2006, Nicol *et al.* 2008, Robertson 1989). This process includes a rate-limiting step where NH_3 is

first oxidized to hydroxylamine (NH_2OH) which is an unstable compound. The key enzyme of the aerobic ammonia oxidation is the ammonia monooxygenase (AMO). It is comprised of three subunits (α-, β- and γ-Amo) which are encoded by the respective genes of the *amoCAB* operon (Arp *et al.* 2007, Klotz *et al.* 1997). The *amoA* gene effectively reflects the phylogeny of ammonia oxidizers; this gene is generally used as a functional marker gene for environmental studies. Neither the phylogeny nor the functional traits of the *amoB* and *amoC* genes have been completely clarified so far (Aakra *et al.* 2001, Purkhold *et al.* 2000, Rotthauwe *et al.* 1997). The oxidation process of the unstable compound NH_2OH into NO_2^- is catalyzed by hydroxylamine oxidoreductase (HAO). It is a multimeric protein which contains at least seven *c*-type hemes and an active-site heme (Arciero and Hooper 1993, Bergmann *et al.* 2005, Hooper and Johnson 1999). The HAO is encoded by the *hao* gene which is only detected in hydroxylamine-oxidizing bacteria like *Nitrosococcus*, *Nitrosospira* and *Nitrosomonas* (Hommes *et al.* 1994, McTavish *et al.* 1993, Schmid *et al.* 2008).

Ammonia oxidation by both bacteria and archaea is widely detected in terrestrial ecosystems, including agricultural soil, grassland, forest and alpine soils (Adair and Schwartz 2008, Boyle-Yarwood *et al.* 2008, Schauss *et al.* 2009). The microbes involved in the ammonia oxidation process are mostly chemolithotrophic and they receive energy through electron exchange when NH_3 is oxidized into NO_3^- (Ferguson *et al.* 2007, Kowalchuk and Stephen 2001). The archaea involved in ammonia oxidation might be chemolithotrophic or mixotrophic (Prosser and Nicol 2008, Xu *et al.* 2012).

The second step in nitrification is the oxidation of NO_2^- into NO_3^- by nitrite oxidoreductase (NXR); so far this gene has been exclusively detected in nitrite oxidizing bacteria such as *Nitrobacter* and *Nitrococcus* (Bothe *et al.* 2000, Daims *et al.* 2001, Jetten *et al.* 1997, Prosser and Embley 2002, Regan *et al.* 2002).

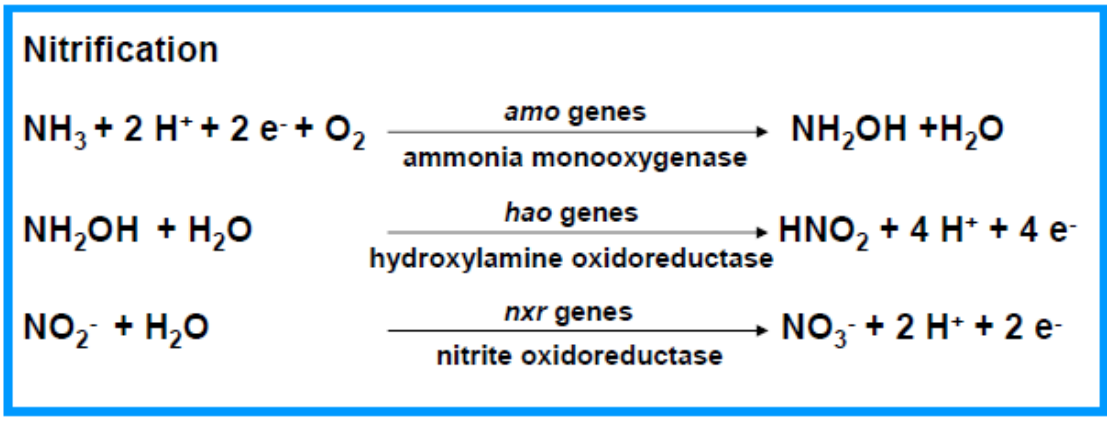

Figure 3 The reaction equations of nitrification. Indications below the arrows describe the enzymes which catalyze respective the oxidation processes; and the indications above the arrows describe the corresponding genes. Modified from Hai (2009).

The NXR of *Nitrobacter* is an integral iron-sulfur molybdoprotein. It consists of at least two subunits, NxrA and NxrB, which are encoded by the *nxrA* and *nxrB* genes, respectively (Lücker *et al.* 2010, Meincke *et al.* 1992, Starkenburg *et al.* 2006).

1.2.2 Denitrification

The end-product of nitrification, NO_3^- is a thermodynamically-favorable N electron acceptor for denitrification (Heylen *et al.* 2006). Denitrification is found in a variety of microorganisms, such as bacteria, archaea and fungi (Bothe *et al.* 2000, Hayatsu *et al.* 2008, Philippot *et al.* 2007, vanSpanning *et al.* 2007, Zumft 1997). Denitrification is the stepwise enzymatic reduction of NO_3^- and NO_2^- to NO, N_2O and N_2 as shown in Figure 4 (Skiba *et al.* 1993, vanSpanning *et al.* 2007, Zumft 1997).

In the first step, NO_3^- is converted to NO_2^- by the membrane-bound (NAR) or the periplasmic nitrate reductase (NAP) (Innerebner *et al.* 2006, Zumft 1997). The NAR consists of three catalytic subunits, which are encoded by *narG*, *narH*, and *narI* genes. The NAP is a heterodimer, which consists of at least two subunits encoded by *napA* and *napB* genes (Philippot 2002, Simpson *et al.* 2010, Zumft 1997).

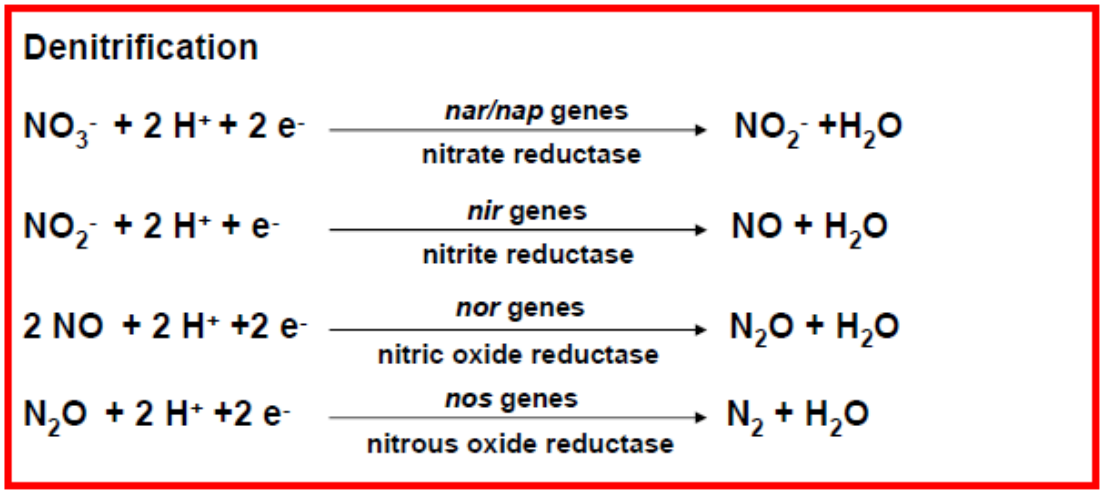

Figure 4 The reaction equations of denitrification. Indications below the arrows describe the enzymes which catalyze the respective reduction processes; and the indications above the arrows describe the corresponding genes. Modified from Hai (2009).

The copper-containing (NirK) and the cytochrome *cd1* (NirS) nitrite reductase are the essential enzymes of NO_2^- reduction, which is the second step in denitrification (Kandeler *et al.* 2006, Philippot 2002). NirK and NirS are homotrimer which have equal functions but are different in structure and prosthetic metal (Glockner *et al.* 1993). NirK has two copper atoms per monomer and NirS has one heme *cd*1 per monomer (Jetten *et al.* 1997, Zumft 1997). No strain has yet been found to harbor both enzymes (Heylen *et al.* 2006). Both genes have been used to detect denitrifiers in environmental samples (Avrahami *et al.* 2002). Niche differentiation of the denitrifying populations has been indicated in different studies (Cole *et al.* 2004). For example, the *nirK* gene is dominant in the rhizosphere, while the *nirS* gene is dominant in the bulk soil (Avrahami *et al.* 2002, Hai *et al.* 2009, Kandeler *et al.* 2006, Sharma *et al.* 2005).

There are three types of nitric oxide reductase (NOR) which use different electron donors involved in the reduction of NO to N_2O: (I) cNOR uses soluble *c*-type cytochromes or pseudoazurin; (II) qNOR uses ubihydroquinone (QH_2) or menahydroquinone (MQH_2); (III) qCu_ANOR uses MQH_2 and cytochrome *c551* (Jüngst and Zumft 1992, Matsumoto *et al.* 2012, Philippot *et al.* 2007, Suharti and

Pouvreau 2007, Zumft 1997).

Nitrous oxide reductase (NosZ), which is encoded by the *nosZ* gene, catalyzes the reduction of N_2O to N_2 (Walker *et al.* 2008). NosZ is a periplasmic homodimeric protein with two subunits which respectively incorporates four copper atoms (SooHoo and Hollocher 1991). The NosZ is sensitive to O_2. Consequently, O_2 has a strong influence on the N_2O/N_2 ratio (Morley *et al.* 2008). Several studies have proved that the N_2O / (N_2O+N_2) ratio was also significantly affected by soil moisture level and soil pH (Ciarlo *et al.* 2008, Čuhel *et al.* 2010).

Due to the influence of N_2O on global warming and the destruction of the ozone layer, the reduction of N_2O is a most important step related to environmental change. Denitrification is therefore one of the most major pathways of N loss in global change (Hofstra and Bouwman 2005, Philippot *et al.* 2007).

1.2.3 The impact of freezing and thawing on nitrification and denitrification

Climatic conditions influence microbial performance in soil. Freezing and thawing in soil disrupts soil aggregates, increases the release of aggregate-protected organic C and causes the death of microorganisms (Bullock *et al.* 1988, Edwards *et al.* 1998). These changes result in an increasing availability of substrate and enhance microbial activity in soil (Sharma *et al.* 2006).

Besides the vegetative period, winter is also important to annual N_2O emissions. Wolf *et al.* (2010) showed that up to 70% of the annual N_2O emissions from agricultural fields might occur in winter. Peak emissions of N_2O from arable soil were detected shortly after thawing. In part, this could be attributed to N_2O being physically trapped in soil aggregates during soil freezing (Dörsch *et al.* 2004, Teepe *et al.* 2001). A large part of N_2O arises from the microbial denitrification processes which were accelerated by frequent freezing and thawing cycles (Öquist *et al.* 2004). Bochove *et al.* (2000)

pointed out that the effect of soil freezing was stronger in small macro-aggregates than in large ones. The reason might be because small macro-aggregates have higher water contents. The ice layer covering the unfrozen water film could be a diffusion barrier which reduces the O_2 supply to the microorganisms and this partly prevents the release of N_2O into atmosphere (Burton and Beauchamp 1994, Goodroad and Keeney 1984, Teepe *et al.* 2001). However, knowledge of the relationship between N_2O emissions and nitrification is limited.

Although increased microbial N turnover can be observed in winter, it is still not well understood how different functional microbial communities respond towards freezing and thawing (Phillips 2008, Ruser *et al.* 1998). A modeling study by De Bruijn *et al.* (2009) implicated freezing and thawing in causing N_2O emissions but that they were not mono-causal. They depended on the amount and quality of available C and N, the amount of microbial biomass and the redox conditions in soil after thawing.

Metagenomic analysis and clone library studies have clearly shown that high concentrations of available C as well as anoxic conditions do not favor the growth of ammonia oxidizers, AOA and AOB (Humbert *et al.* 2010, Liebner *et al.* 2008, Yergeau *et al.* 2010). Müller *et al.* (2002) showed that the increased NH_4^+ and NO_3^- concentrations in the freezing period were associated with peak N_2O emissions in the following thawing period. On the other hand, Sharma *et al.* (2006) observed an increase in the abundance of *napA* and *nirK* genes; straight after thawing, these encode for the NO_3^- and NO_2^- reductases respectively.

Additionally, the presence of soluble C in soil is necessary to induce N_2O production during freezing and thawing events (Christensen and Christensen 1991). Plant residues may play an important role in the regulation of N_2O emissions in winter since freeze-thaw stress causes them to release organic compounds. However, the type of plant residues showed only limited effects on the N cycle (Chèneby *et al.* 2010). In contrast to permafrost soils, increased nitrification rates were detected in soils from

moderate climatic zones after thawing. Schleper *et al.* (2005) and Valentine (2007) presumed that archaea are more tolerant to stress conditions than bacteria. The abundance of bacterial ammonia oxidizers (AOB) are decreased by freeze-thaw stress, whereas their archaeal counterparts (AOA) are increased. Therefore, archaea is more worth to analysis the microbial community shift after freezing and thawing.

1.3 Methods to assess microbial communities

Conventional techniques in microbial ecology include the cultivation of microbes on artificial media under laboratory conditions in order to identify and quantify microorganisms. However, only 1% of the microorganisms from the environment can be isolated by using these methods (Amann *et al.* 1995). Therefore, information about biological variety, composition of microbial populations and specific activity in soils is limited. In the last few decades molecular methods have been developed to overcome this problem and to study microbial communities based on extracting nucleic acids directly from environmental samples without further cultivation of the corresponding microbes (Adair and Schwartz 2008, Kandeler *et al.* 2006).

1.3.1 Extracting DNA from soil samples

DNA from soil can be extracted from the cells of microorganisms separated from soil samples or directly extracted from soil samples (C.Yeates *et al.* 1998, Kauffmann *et al.* 2004). A culture-independent rapid protocol for the extraction of total nucleic acids from environmental samples was described in Griffiths *et al.* (2000). DNA and RNA from soil samples were co-extracted by phenyl-chloroform extraction and subsequently enzymatic digestion. Töwe *et al.* (2011) improved the protocol to provide a simultaneous and reproducible extraction and separation of DNA and RNA. The improved protocol raised the quality and quantity of DNA and RNA which favors the reliable analyses of gene transcript copy numbers and their diversity patterns. In

the last few years, more and more commercial nucleic acid extraction kits have become available, which allow for a rapid and highly-standardised but often inefficient DNA or RNA extraction.

1.3.2 Abundance of functional microbial groups

In order to determine the abundance of genes in soil samples, quantitative real-time PCR (qPCR) is commonly used. The use of qPCR enables the specific detection of PCR products in real-time during the exponential amplification stage of the reaction. It integrates the amplifications and analysis steps by monitoring the DNA production during each amplification cycle. Fluorescent technology is used for quantifying the product at the end of each amplification cycle in the PCR reaction (Saleh-Lakha *et al.* 2005).

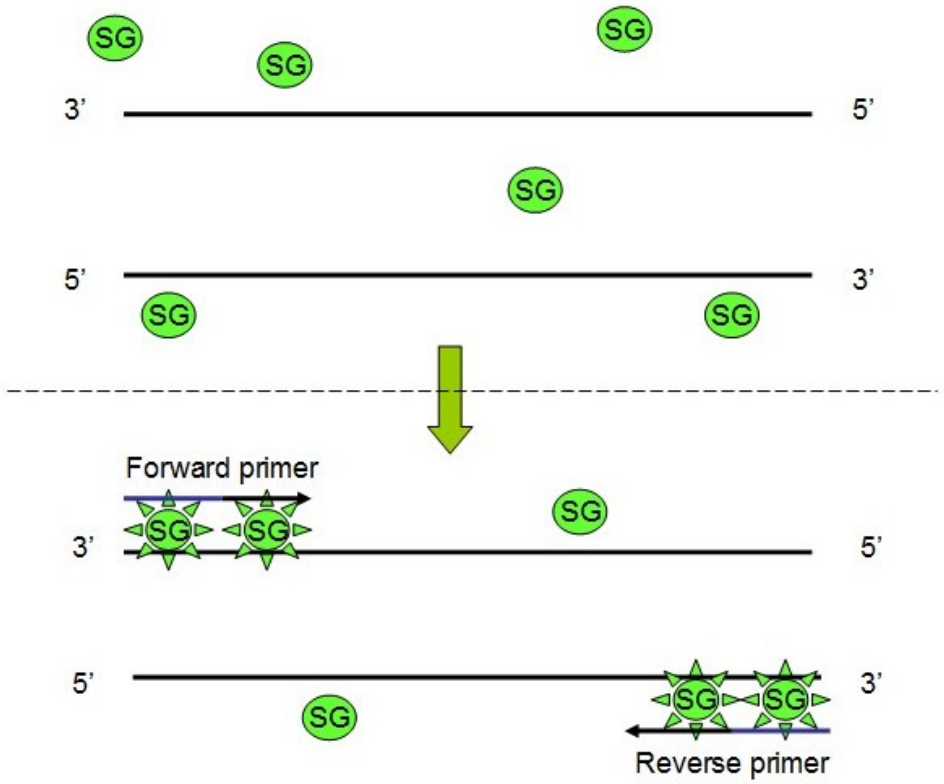

Figure 5 The mechanism of SYBR Green (SG). SG starts fluorescing as soon as it intercalates double-stranded products. Modified from Gschwendtner (2010).

Several types of fluorescent markers can be chosen, such as SYBR Green (SG), Taqman and molecular beacons. Due to the ability to perform melt curves and the less assay setup and costs, SG has frequently been used. The mechanism of SG is shown in Figure 5: SG intercalates all double-stranded DNA in the samples and binds to each new copy of double-stranded DNA generated during the amplification process, then starts fluorescing. However, unspecific products may occur, for example, primer dimers, as a result of the intercalation with any double-stranded product (Wittwer *et al.* 1997).

In qPCR, the threshold cycle (CT) method is usually used to obtain quantitative information based on a comparison of the samples with external DNA standards. These DNA standards are serially-diluted and used for calibration (Röder *et al.* 2010). However, the performance of the dissociation curve analysis and the melting peaks of the amplicons allows for distinct differentiation between the targeted products and undesired contaminants and primer dimers (Rasmussen *et al.* 1998). In case of environmental samples, divergences in the GC content of a specific gene may lead to the formation of multiple peaks (Sharma *et al.* 2007).

1.3.3 The diversity of functional microbial groups

Genetic fingerprinting comprises a set of methods that are appropriate for the assessment of microbial community structure, such as terminal-restriction fragment length polymorphism (T-RFLP), clone library, and denaturing gradient gel electrophoresis (DGGE) (Kitts 2001, Maukonen and Saarela 2009). Compared to other methods, T-RFLP as a comparative community analysis method shows higher sensitivity and can detect minor shifts of communities. Therefore, it is used to analysis changes of community structure under different conditions (Sørensen *et al.* 2009). T-RFLP is based on the restriction end nuclease digestion of fluorescently end-labeled PCR products. The digested products are detected on an automated sequence analyzer. The method provides distinct profiles, fingerprints, which are

dependent upon the species composition of the sample communities (Bukovská *et al.* 2010).

T-RFLP can be used to analyze a large number of samples. It is a useful tool for the initial screening for similarities and differences between different bacterial soil communities (Kirk *et al.* 2004). It was reported that the use of terminal restriction fragment (T-RF) peak number can be a suitable measurement of the species richness in bacterial communities (Dunbar *et al.* 2000). However, an individual T-RF does not always represent an individual species or genus. Furthermore, different restriction enzymes can provide different levels of phylotype richness within a community or different trends between communities. Despite its limitations in identifying individual species or in describing species richness, the T-RF method remaims is useful for detecting compositional differences in complex soil communities (Kitts 2001, Nakatsu 2007).

1.4 Aims and hypothesis

Climate change, especially freezing and thawing actions influence the nitrification and denitrification processes in soil. Soil thawing is often accompanied by nutrients release and emission of greenhouse gases such as N_2O, NO. N_2O emissions from cultivated soil are estimated to account for more than 75% of the total global anthropogenic emissions. The overall aim of this thesis was to improve the knowledge of freeze-thaw effects on N cycle processes in arable soils under different treatments: I) the effects of plant residues with different carbon and nitrogen quantities; II) the effects of the presence of Cu^{2+} in soil.

It was assumed that:

1) Litter quality has a pronounced effect on nitrification and denitrification during freezing and thawing.

2）The influence of different qualities and quantities of plant litter-derived C is less pronounced compared to plant-derived N.

3) Cu^{2+} contamination has a negative impact on the abundance and diversity of nitrifiers and denitrifiers, and reduces the transformation rates of nitrification and denitrification during freeze-thaw cycles.

To prove these hypotheses, three microcosm experiments were conducted. In two of experiment, arable soil was amended with legume-grass mixtures or wheat straw, with similar C but different N content. In the second experiment, soil samples with similar N but different C content were exposed to a freeze-thaw cycle. A quantitative analysis of *amoA*, *nirK*, *nirS* and *nosZ* gene abundances in the soils was carried out by using qPCR. Water extractable organic carbon (WEOC), total nitrogen (TNb) and mineral N fractions (NO_3^- and NH_4^+) were also measured to determine whether freeze-thaw cycles influenced nitrification and denitrification when there was different C and N input.

Due to the toxicity of Cu^{2+} for many prokaryotes and eukaryotes, the effects of re-mobilized Cu^{2+} on microbial performance were investigated in an arable soil, especially after freezing and thawing. The influence of Cu^{2+} as a contamination of nitrifiers and denitrifiers was examined based on the abundance of the functional genes, *amoA*, *nirK*, *nirS* and *nosZ*. Additionally, the quantities of mineral N fractions (TNb, NO_3^- and NH_4^+) and WEOC were measured after freezing and thawing. The *amoA* (AOA) and *nosZ* genes were chosen to represent the nitrification and denitrification community shift during the freezing and thawing event and were identified by T-RFLP.

2 Material and methods

2.1 Soil description

The soil used was taken from the research farm, Klostergut Scheyern, which is located about 40 km north of Munich in Bavaria, Germany (48°30.0′ N; 11°20.7′ E). The mean annual temperature of this site is 7.4 °C and the average annual precipitation is 833 mm yr^{-1}. During the last few decades, farming management on this research farm has shifted from a conventional farming management to an integrated management using minimum tillage and continuous crop cover (Schröder *et al.* 2002). An arable field farm (A15, 4.75 ha) was chosen for soil collections in September 2008, July 2009 and May 2010. This field farm performed integrated crop production (Sommer *et al.* 2003). During the soil collection times of the investigation, the field was cropped with potatoes in September 2008 and November 2010 and winter wheat in October 2009 in a rational crop rotation. The upper 20 cm depth of the ploughed soil horizon (Cambisol) was collected at the three sampling time points mentioned above and its composition was characterized as follows: 22% clay, 36% silt and 42% sand; C/N ratio of 10.0; pH value 5.8 (0.01 M $CaCl_2$).

2.2 Experimental design and sampling

Three experiments were designed to investigate freeze-thaw effects on the soils with different treatments. The first freeze-thaw experiment (E1) was carried out with different amendments of legume-grass mixtures or wheat straw under similar C quantities but with different amounts of N; the second experiment (E2) used the same amendments with a similar N quantity but with differences in the C content of the soil; the third experiment (E3) treated soil with/without additional $CuCl_2$ as a contamination under same background of legume-grass mixtures. The amount of amendment in E3 was as the same as in E1.

In all the three experiments, the soil was air-dried and then put through a 2 mm sieve to remove stones, crop residues and roots (Xiao *et al.* 2010). Legume-grass mixtures and *Triticum aestivum* were obtained from the research farm to be organic amendments. Legume-grass mixtures and wheat straw exhibit similar C contents at 421g kg^{-1} and 436 g kg^{-1} respectively but differ considerably in their C/N ratios which are 10.1 and 126.6 respectively, resistance and biodegradability (Müller *et al.* 1998, Nicolardot *et al.* 2007, Wang *et al.* 2004). The residues were air-dried and 2 mm ball-milled (Retsch, Germany) before use.

The soil and its respective plant residue amendment which had been milled to a size of 2 mm were thoroughly mixed and placed in metal cylinders of 4 cm height and a volume of 100 cm^3. All the samples were pre-incubated at 10 °C with 50% maximum water-holding capacity for one week. Half of the thus-treated microcosms were frozen after a pre-incubation period for three days at -20 °C, followed by thawing at 10 °C; the other half were incubated at a constant temperature of 10 °C to act as controls. Samples were taken at the day before freezing (day 0, labeled as T0), and on the first, third and seventh days after thawing (day 4, labeled as 'T1'; day 6, labeled as 'T2' and day 10, labeled as 'T3'). These were prepared for chemical and molecular analyses as shown in Figure 6.

2.2.1 Influence of freeze-thaw on amended soil with different N quantities (E1)

Legume-grass mixtures and wheat straw were added to the soil samples with similar amounts of C but different N quantities, corresponding to 222 dt C ha^{-1}, 1.8 dt N ha^{-1}, in order to investigate how the different N contents of the plant residues affected the microbial communities in soils after freeze-thaw treatment. An amendment ratio of 2 g plant residue -either legume-grass mixtures or wheat straw-per 50 g soil was used in E1. Four independent replicates were prepared for each plant residue under both freeze-thaw and non freeze-thaw (control) conditions for each sampling time point. All the samples were pre-incubated for two weeks (see Figure 6).

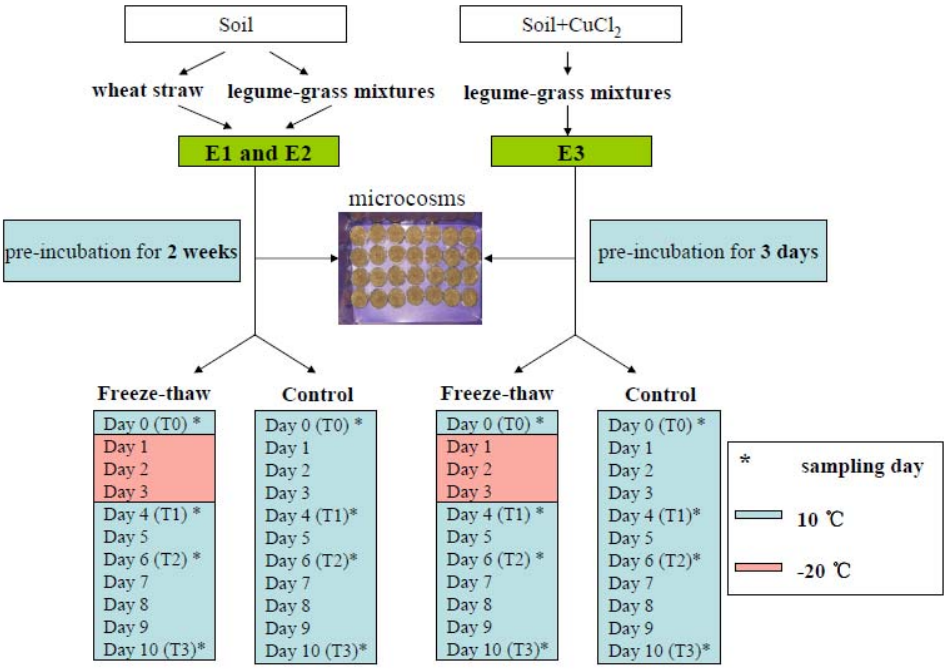

Figure 6 Diagram of the experimental design and sampling time points.

2.2.2 Influence of freeze-thaw on amended soil with similar N quantities (E2)

In order to investigate how soils amended with different plant residues under similar N quantities reflected freeze-thaw effects, comparable amounts of N were added. 4 g wheat straw, corresponding to 444 dt C ha^{-1}, 3.6 dt N ha^{-1}, or 0.3 g legume-grass mixtures, corresponding to 37 dt C ha^{-1}, 3.6 dt N ha^{-1}, were added into the microcosms as described above. Five independent replicates were prepared for each plant residue under both freeze-thaw and control conditions for each sampling time point. All samples were pre-incubated for two weeks.

2.2.3 Influence of freeze-thaw on Cu^{2+} contaminated soil with same N quantities (E3)

The aim of E3 was to investigate and compare the influence of Cu^{2+} contamination and freeze-thaw cycles to the diversity and abundance of the microbial communities in soil. Copper (II) chloride ($CuCl_2$) was added as a contaminant into sieved soil with

a final concentration of 150 mg kg^{-1} soil (Fernández-Calviño *et al.* 2010). After one week homogenization, the legume-grass mixtures were added as the additional C and N source, corresponding to 222 dt C ha^{-1}, 1.8 dt N ha^{-1} in each soil sample as described above. Due to the fast degradation of the legume-grass mixtures found in E1 and E2, the pre-incubation time for E3 was shortened from two weeks to three days. Five independent replicates were prepared for each treatment and each sampling time point.

2.3 Chemical analyses of soils

2.3.1 Soil sampling and soil extraction

Samples from each sampling time point were separated into three parts for different analysis objectives. The first aliquots of the soil samples were immediately extracted with 0.01 M $CaCl_2$ in an overhead shaker for 30 minutes at room temperature. The ratio of soil/$CaCl_2$ was 1:2 (wt/wt) according to Zsolnay (2003). The supernatant was filtered through a 4 - 7 µm Whatman 595 ½ filter papers (Whatman, Germany) after sedimenting for 10 minutes. The filtrated solutions were stored at -20 °C until used for soil chemical measurements. The second aliquots of the soil samples (2 g) were dried at 100 °C overnight for measuring the water content. The remaining aliquots of the soil samples were stored at -20 °C for molecular biology analyses.

2.3.2 Water extractable organic carbon (WEOC), total nitrogen (TNb), ammonium (NH_4^+), nitrate (NO_3^-) and Cu^{2+} analyses

Both WEOC and TNb were measured in the $CaCl_2$ extracts by using a DIMATOC 100 automatic analyzer (DIMATEC, Germany). WEOC was determined as CO_2 by infrared absorption after combustion at 850 °C, while TNb in the extracts was measured by chemo-luminescence detection after combustion at 850 °C. The results of WEOC and TNb concentrations were analyzed in weighted soil mass in the form of

dry matter and expressed as μg C g^{-1} soil and μg N g^{-1} soil, respectively. All analyses were performed in technical triplicates.

The concentrations of NH_4^+ and NO_3^- in the $CaCl_2$ extracts were measured using the Nanocolor® Ammonium 3 and Nanocolor® Nitrat 50 kit (Merck, Germany) according to the manufacture's instruction in E1. An automated continuous flow analyzer Skalar5521 (Skalar, Holland) for NH_4^+ and NO_3^- quantification was used in E2 and E3. The $CaCl_2$ extracts were diluted with 0.01 M $CaCl_2$ solution and the analyses were carried out in technical triplicates.

The Cu^{2+} concentrations of the soil samples in the $CaCl_2$ extracts were measured using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) on an ICP-AES "Optima 7300" system (Perkin Elmer, Germany) by Prof. Michalke Bernhard (Institute of Ecological Chemistry, Helmholtz Zentrum München). The extracted solutions from soil samples were diluted with Milli-Q water ten times before being measured. Sample injections were carried out using a peristaltic pump equipped with an 'anti-pulse-head' (SPETEC, Germany) connected to a nebulizer with a cyclon spray chamber. The measured spectral element line for Cu^{2+} was 327.393 nm. The radio frequency power was set to 1000 W, the plasma gas was 15 L Ar min^{-1} and the nebulizer gas was 0.6 L Ar min^{-1}. Results were calculated using a computerized lab-data management system, relating the sample measurements to calibration curves, blank determinations, control standards and the weight of the digested samples (Schramel 1983, 1988).

2.4 Molecular analyses of soil microbial communities

2.4.1 DNA extraction and quantification

DNA was extracted from 0.5 g soil by phenol-chloroform extraction following the methods of Griffiths *et al.* (2000) in both E1 and E2. The solutions used in this method are listed in Table 1. In brief, 0.5 g soil was mixed with 0.5 ml of solution A and 0.5 ml solution B in a Lysing Matrix E tube (MP Biomedicals, France). The samples were lysed for 30 seconds using a high-throughput tissue homogenizer Precellys®24 (Bertin Technologies, France) and centrifuged at 16,100 g for five minutes at 4 °C. The aqueous layer was removed and mixed with an equal amount of solution C. After five minutes of centrifugation at 4 °C, the same volume of solution D was added to the aqueous phase. The mixture was incubated for 2 hours on ice for nucleic acid precipitation and then centrifuged for 10 min at 4 °C. The nucleic acid pellet was washed in an ice-cold 70% ethanol solution (solution E) and centrifuged for ten minutes at 4 °C. After discarding the ethanol, the pellet was air-dried and re-suspended in 50 µl of nuclease-free water.

Table 1 Solutions used in DNA extraction.

Solution	Chemicals	Concentration
A	a. hexadecyltrimethylammonium bromide (CTAB) :	
	CTAB	0.1 g ml^{-1}
	NaCl	0.0409 g ml^{-1}
	b. potassium phosphate buffer (pH 8)	240 mM
	1 M K_2HPO_3 : 1 M KH_2PO_4	47:3
	Mix a and b in equal volumes to be the solution A.	
	Add 10µl β-Mercaptoethanol to 1 ml solution A before use.	
B	Phenol : chloroform : isoamyl alcohol (pH 8)	25:24:1
C	Chloroform : isoamyl alcohol	24:1
D	Polyethylene glycol (PEG)	10%
	NaCl	1.2 M
E	Ethanol	70%
F	DEPC-H_2O	0.1%

Afterwards, the yield of the extracted DNA was determined by Nanodrop1000 (PeqLab, Germany) and the quality was evaluated by calculating the ratio of A260/A280. The DNA was stored at -20 °C until further use (Töwe *et al.* 2011).

In order to remove any possible PCR inhibitors during the DNA extraction from Cu^{2+} contaminated soil, the NucleoSpin® Soil kit (Macherey-Nagel, Germany) was used in E3 (Töwe *et al.* 2011). DNA was extracted from 0.5 g soil according to the protocol of the manufacturer. Cell lysis was performed by using the Precellys®24 Homogenizer (Bertin Technologies, France). Extracts were determined by Nanodrop1000 (PeqLab, Germany) for DNA yield and purity. DNA samples were stored at -20 °C until use.

2.4.2 Quantitative real-time PCR (qPCR) assay

A Power SYBR® Green PCR Master Mix (Applied Biosystems, Germany) was used as a fluorescing dye to quantify the absolute quantity of *amoA* AOA, *amoA* AOB, *nirK*, *nirS* and *nosZ* genes. This was carried out in triplicate on the ABI Prism 7300 Cycler (Applied Biosystems, Germany) (Hai *et al.* 2009). The compositions of each PCR reaction are described in Table 2.

Table 2 The compositions of PCR reactions.

	amoA (AOA) (µl)	*amoA* (AOB) (µl)	*nirK* (µl)	*nirS* (µl)	*nosZ* (µl)
DNA template	2	2	2	2	2
DMSO	-	-	0.625	0.625	-
3% BSA	0.5	0.5	0.5	0.5	0.5
10 µM Forward primer	0.5	0.75	0.5	0.5	0.5
10 µM Reverse primer	0.5	0.75	0.5	0.5	0.5
DEPC-H_2O	9	8.5	8.375	8.375	9
Power SYBR® Green PCR Master Mix	12.5	12.5	12.8	12.8	12.5
Total volume	25	25	25	25	25

Table 3 shows the standards which were prepared by a series of PCR, ligation and transformation using the TA Cloning® Kit (Invitrogen, Germany) and pCR®2.1 vector. A dilution series of 1:5, ranging from 10^1 to 10^6 gene copies μl^{-1}, were used to generate standard curves for each target gene (Töwe *et al.* 2010). A DNA dilution series of 1:2, 1:4, 1:8, 1:16 and 1:32 was tested to determining the optimal dilution ratio in order to avoid the possible inhibitory effects of co-extracted humic substances on qPCR.

Table 3 Standards and primers used for PCR quantification of different functional genes.

Target gene	Source of standard	Amplicon size (bp)	Primer	Sequence of primer
amoA AOA	Fosmid clone 54d9 (Treusch *et al.* 2005)	624	19F (Leininger *et al.* 2006) CrenamoA616r48x (Schauss *et al.* 2009)	Forward: 5'-atg gtc tgg ctw aga cg-3' Reverse: 5'-gcc atc cab ckr tan gtc ca-3'
amoA AOB	*Nitrosomonas* sp. (Pinck *et al.* 2001)	500	amoA 1F amoA 2R (Rotthauwe *et al.* 1997)	Forward: 5'-ggg gtt tct act ggt ggt-3' Reverse: 5'-ccc ctc kgs aaa gcc ttc ttc-3'
nirK	*Azospirillum irakense 11586*	164	nirK 876 (Henry *et al.* 2004) nirK 5R (Skiba *et al.* 1993)	Forward: 5'-aty ggc ggv cay ggc ga-3' Reverse: 5'-gcc tcg atc agr ttr tgg-3'
nirS	*Pseudomonas stutzeri*	413	nirS cd3af (Michotey *et al.* 2000) nirS R3cd (Brookes 1995)	Forward: 5'-gts aac gts aag gar acs gg-3' Reverse: 5'-gas ttc ggr tgs gtc ttg a-3'
nosZ	*Pseudomonas fluoreszenz C7R12*	267	nosZ2F nosZ2R (Henry *et al.* 2006)	Forward: 5'-cgc rac ggc aas aag gts mss gt-3' Reverse: 5'-cak rtg cak sgc rtg gca gaa-3'

In this research, a regularly increased cycle threshold value (Ct-value) was found between 1:16 and 1:32 dilutions. In order to simplify the calculation, a dilution ratio of 1:30 was chosen as the standard for diluting all the DNA samples.

In each qPCR run, samples and standards were performed in triplicate and at least four negative controls were included in each qPCR plate. All the qPCR runs started with an initial enzyme activation step performed at 95 °C for 10 min. The subsequent steps were different for each gene as described in Table 4. Afterwards, the dissociation stage was attached as follows: 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The results of qPCR were reported as gene copy numbers per gram of dry soil. The amplification efficiency (Eff) was calculated by using the formula $Eff = [10^{(1/slope)}-1]$ and the specificity of the amplification products was confirmed by melting-curve analysis and agarose gels.

2.4.3 Terminal restriction fragment length polymorphism (T-RFLP) assay

The shift of microbial community was analysed using T-RFLP. These were performed with one functional gene of each examined process: in nitrification it was the archaea *amoA* gene and in denitrification it was the *nosZ* gene. Similar primers and the thermal profile were used as described above, except that 30 and 35 cycles instead of 40 cycles were performed. The forward primers were labeled with 5'- FAM (6-carboxyfluorescein). PCR amplifications were carried out in triplicate as shown in Table 5. DNA from a Fosmid clone 54d9 and *Pseudomonas fluoreszenz* C7R12 were used as the positive control for *amoA* (AOA) and *nosZ* gene, respectively (Table 3). PCR reaction mixture with all components except a DNA template was used as negative control. PCR products were purified with Qia quick PCR Purification Kit (Qiagen Inc, Germany) and quantified with Nanodrop1000 (PeqLab, Germany).

Table 4 Thermal profiles used for the qPCR quantification of different functional genes.

Target gene	Thermal cycling profile	cycles
amoA AOA	94 °C - 45 s / 55 °C - 45 s / 72 °C - 45 s	40
amoA AOB	94 °C - 45 s / 58 °C - 45 s / 72 °C - 45 s	39
nirK	95 °C - 15 s / 63 °C - 30 s / 72 °C - 30 s	5[a]
	95 °C - 15 s / 58 °C - 30 s / 72 °C - 30 s	40
nirS	94 °C - 45 s / 57 °C - 45 s / 72 °C - 45 s	39
nosZ	95°C - 15s / 65°C - 30 s / 72 °C - 30 s	5[a]
	95°C - 15s / 60°C - 30 s / 72 °C - 30 s	40

[a] Touchdown: -1°C cycle^{-1} 30s

Afterwards, 200 ng of purified PCR products were digested using restriction enzymes Tsp509I (*amoA* AOA), and HpyCH4V (*nosZ*) (New England Biolabs, Germany) according to the manufacturers' protocols. The enzyme selections were based on *in situ* T-RFLP by using the programme of a restriction endonuclease picker (Bannert *et al.* 2011, Collins and Rocap 2007). Afterwards, digested PCR products were purified by using MinElute Reaction Cleanup kit (Qiagen Inc, Germany). The purified digestion products were quantified with the Nanodrop (PeqLab, Germany) and subsequently diluted to 5 ng µl^{-1}.

1 µl of each purified digestion product was then mixed with 13 µl Hi-Di™ Formamide (Applied Biosystems, USA) containing a 800-fold dilution of a 6-carboxy-X-rhodamine labeled MapMarker 1000 ladder (Bio-Ventures, USA.). The mixture was denatured for five minutes at 95 °C in a thermocycler (Biometra, Germany) and cooled on ice immediately. DNA fragments of different sizes were separated and detected using Applied Biosystems capillary electrophoresis platforms 3730 DNA analyzer (Applied Biosystems, Germany). Electrophoresis was performed with POP-7™ polymer in a 50 cm capillary array under the following conditions: 10 seconds of injection time, 2 kV injection voltage, 7 kV run voltage, 66 °C run temperature, and 63 min for analysis time (Töwe *et al.* 2011). Fluorescence emissions from the labeled DNA sequence fragments were collected simultaneously and spectrally separated by a spectrograph.

Table 5 PCR protocols used for T-RFLP of different functional genes.

Genes	PCR protocol	
amoA(AOA)	5 µl	10 × Buffer
	2.5 µl	25 mM $MgCl_2$
	1 µl	10 µM Fam-Forward-Primer
	1 µl	10 µM Reverse-Primer
	5 µl	10 mM dNTP
	1 µl	3% BSA
	2 µl	DNA template
	0.5 µl	TopTaq DNA Polymerase (5000 units ml^{-1})
	32 µl	DEPC-H_2O
nosZ	5 µl	10 × Buffer
	1 µl	10 µM Fam-Forward-Primer
	1 µl	10 µM Reverse-Primer
	2.5 µl	10 mM dNTP
	2 µl	DNA template
	0.25 µl	TopTaq DNA Polymerase (5000 units ml^{-1})
	38.25 µl	DEPC-H_2O

The height of the peak for each fragment was expressed as a percentage in each T-RFLP profile and used to estimate the relative abundance of each terminal restriction fragment in each sample (Culman *et al.* 2009). The electropherograms were analyzed using the GeneMapper® 3.5 software package (Applied Biosystems, Germany).

2.5 Statistical analyses

2.5.1 Soil chemical and molecular analyses

Soil chemical and molecular data from each experiment were analyzed using a multi-factorial analysis of variance (ANOVA, SPSS 11.5) with the following independent variables: freeze-thaw, legume-grass mixtures and wheat straw amendments in E1 and E2, Cu^{2+} contamination in E3 and sampling time points, T0, T4, T6, T10. Normal distributions of the variables were checked using the

Kolmogorov-Smirnov test and box-plot analysis, whereas the homogeneity of variances was analyzed using the Levene test. Molecular analyses results were first log transformed and then the distributions and homogeneity of the variables were checked. If there was homogeneity of the variances, then Tukey's Honestly Significant Difference (HSD) was applied for pair-wise comparison of means; if not, a Dunnett-T3 test was applied. The significant difference was set to the 0.05 level ($P < 0.05$).

2.5.2 T-RFLP data analysis

The T-RFLP assay results were analyzed using multivariate analyses, which were based on a type of descriptive discriminated analysis (Culman *et al.* 2009). The raw data were evaluated and analyzed using GeneMapper® 3.5 software. The processed data included the size of the fragment, peak height and peak area; there were further processed by T-RFLP analysis expedited (T-REX, http://trex.biohpc.org) for each experiment. Any fragments with less than 50 bp and the fragments with low abundance, i.e. less than 5% of the total intensity, were removed. To allow for the different factors of each experimental setting, with or without copper contamination, freeze-thaw or control treatment, sampling days, 14 subgroups were analyzed by using supervised learning methods, which optimizes the between-group variance (BGA) based on correspondence analysis. Due to the relatively small number of replicates (n = 5), a method which is equivalent to the diagonal discriminated analysis was used. A permutation test was performed to test the global effect of the factor combination. Briefly, for each analysis term, 999 random permutations of the raw data were conducted to obtain *P* values. In the case of a significant result ($P < 0.05$) being found, tests were performed comparing all pairs of group; the *P* values were adjusted for multiple comparisons using the method of Hommel (1988). All multivariate analyses were done by Dr. Gerhard Welzl within the R software environment for statistical computing (http://www.R-project.org).

3 Results

3.1 Influence of freeze-thaw cycles on ammonia oxidizers and selected denitrifiers in soils amended with litter differing in the amount of nitrogen (E1)

3.1.1 Soil chemical analyses

The samples had similar C (1.7×10^4 µg C g^{-1} soil) contents but different total N contents in the amendments. In the legume-grass mixtures it was 1.6×10^3 µg N g^{-1} soil but in the wheat straw it was 1.4×10^2 µg N g^{-1} soil, significantly higher values of WEOC, TNb, NH_4^+ and NO_3^- contents were detected in the samples amended with legume-grass mixture as shown in Table 6. These were found at all sampling time points, independent of freezing and thawing cycles. The WEOC content of the legume-grass mixtures was 4.0 - 5.4 times higher compared with that of the wheat straw amendment (see Figure 7 A). The concentrations of TNb, NH_4^+ and NO_3^- were also much higher in legume-grass mixtures at 105.0 - 136.9 µg TNb, 54.0 - 78.2 µg NH_4^+ and 54.0 - 118.1 µg NO_3^- per gram of soil, respectively, than in wheat straw amended samples at 1.9 - 2.3 µg TNb, up to 0.16 µg NH_4^+ and 3.2 - 5.5 µg NO_3^- per gram of soil, respectively (see Figure 7 B). The NH_4^+ from soils amended with legume-grass mixtures was especially increased by 339 - 1694 times compared with the soils mixed with wheat straw (see Figure 7 C).

Samples of soil amended with the legume-grass mixtures which had been subjected to freezing and thawing showed significantly higher amounts of WEOC at 151.5 - 232.9 µg g^{-1} soil than in the non-frozen control samples at 126.3 - 148.4 µg g^{-1} soil. Additionally, significant effects were detected on sampling times and the interaction between time and freeze-thaw effect in legume-grass mixture amended samples on WEOC contents (see Table 6).

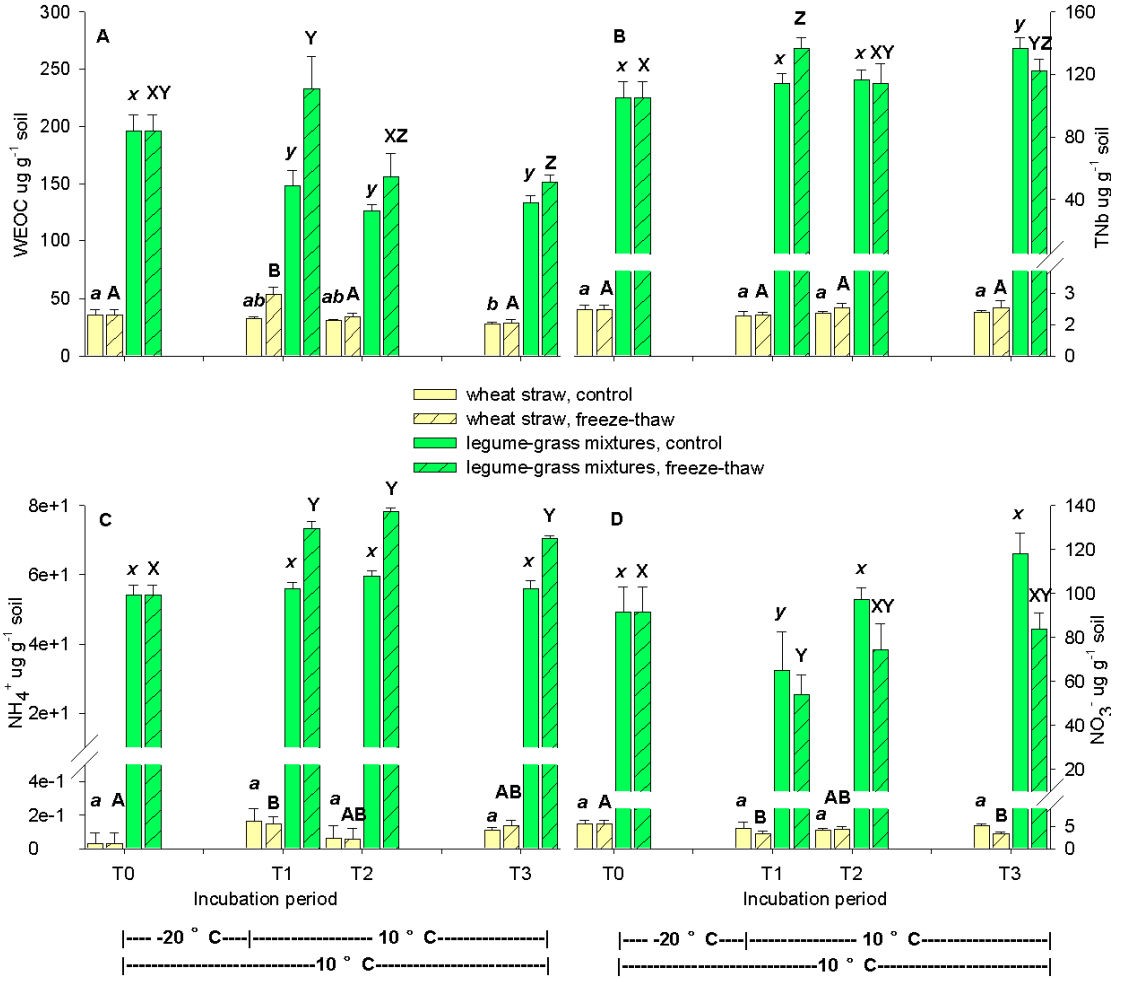

Figure 7 Amounts of WEOC (A), TNb (B), NH_4^+ (C) and NO_3^- (D) in E1. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n = 4). Different letters display significant differences at the different time points.

In detail, the WEOC content showed a significant decrease from T0 (195.8 µg g^{-1} soil) to T3 (133.6 µg g^{-1} soil) in non-frozen control samples; whereas, in the freeze-thaw treated samples the WEOC content increased from T0 (195.8 µg g^{-1} soil) to T1 (232.9 µg g^{-1} soil), and subsequently decreased during the thawing period (T2 and T3).

No significant effect of freezing and thawing was observed for TNb in samples which had been amended with the legume-grass mixture; the values ranged from 114.3 to 136.9 µg g^{-1} soil (see Figure 7 B). However, the TNb values significantly increased from T0 (105.0 µg g^{-1} soil) to T3 (freeze-thaw, 122.2 µg g^{-1} soil; control, 136.9 µg g^{-1}

soil). Samples which had been subjected to freezing and thawing, revealed 1.25 - 1.31 times higher NH_4^+ concentrations than non-frozen control soil samples. Sampling time points showed a significant increase in NH_4^+ concentrations from T0 (54.0 μg g^{-1} soil) to T3 (70.5 μg g^{-1} soil) in samples which had been subjected to freezing and thawing. There was no significant difference detected in sampling time in non-frozen control samples (see Table 6).

In contrast to the WEOC and NH_4^+ contents, NO_3^- content displayed 1.2 to 1.4 times higher values in non-frozen control samples than in samples which had been subjected to freezing and thawing (see Figure 7 D) in samples that had been amended with the legume grass mixture.

Table 6 Statistical evaluations of the chemical parameters of the soil using ANOVA in E1. The *p* values describe the impacts and interactions of sampling time point, soil treatments and amendments on soil WEOC, TNb, NH_4^+ and NO_3^-. Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values			
	WEOC	TNb	NH_4^+	NO_3^-
Total				
Time (T0, T1, T2,T3)	0.00 *	0.00 *	0.00 *	0.00 *
Treatment (freeze-thaw, control)	0.00 *	0.60	0.00 *	0.00 *
Amendment (legume-grass mixtures, wheat straw)	0.00 *	0.00 *	0.00 *	0.00 *
Time × treatment	0.00 *	0.00 *	0.12	0.06
Time × amendment	0.00 *	0.00 *	0.00 *	0.00 *
Treatment × amendment	0.00 *	0.74	0.00 *	0.00 *
Time × treatment × amendment	0.00 *	0.00 *	0.11	0.05
Legume-grass mixtures amendment				
Time (T0, T1, T2,T3)	0.00 *	0.00 *	0.00 *	0.00 *
Treatment (freeze-thaw, control)	0.00 *	0.67	0.00 *	0.00 *
Time × treatment	0.00 *	0.01 *	0.13	0.07
Wheat straw amendment				
Time (T0, T1, T2,T3)	0.00 *	0.98	0.02 *	0.01 *
Treatment (freeze-thaw, control)	0.00 *	0.11	0.35	0.43
Time × treatment	0.00 *	0.56	0.31	0.23

Moreover, different sampling time points showed significant differences of NO_3^- concentrations (see Table 6). In detail, NO_3^- concentrations decreased from T0 (91.5 µg g^{-1} soil) to T1 (freeze-thaw, 54.0 µg g^{-1} soil; control, 65.2 µg g^{-1} soil) and again increased until T3 (freeze-thaw, 83.8 µg g^{-1} soil; control, 118.1µg g^{-1} soil).

In the wheat straw amended soil samples, a significant influence of freeze-thaw cycles was only detected for WEOC content which increased from 36.3 µg to 54.2 µg g^{-1} soil at T1, whereas, the control sample values ranged around 30 µg g^{-1} soil. Significant effects on sampling time points and the interaction between time and freeze-thaw stress were also visible for WEOC contents (see Table 6). In brief, a significant decrease from T0 (36.3 µg g^{-1} soil) to T3 (28.3 µg g^{-1} soil) was shown in the non-frozen control samples; whereas, significantly higher values were detected at T1 (54.2 µg g^{-1} soil) in the freeze-thaw treated soil samples.

Furthermore, significant effects of sampling time points were also detected in soil samples amended with wheat straw on NH_4^+ and NO_3^- contents (see Table 6). In detail, a difference in the NH_4^+ was detected between T0 (0.03 µg g^{-1} soil) and T1 (0.15 µg g^{-1} soil) in freeze-thaw treated samples. A significant decrease was shown in NO_3^- content from T0 (5.5 µg g^{-1} soil) to T3 (3.2 µg g^{-1} soil) under freezing and thawing stress. However, no significant difference was detected in the NH_4^+ and NO_3^- contents in non-frozen control samples among the sampling time points.

3.1.2 Abundances of ammonia oxidizers and nitrite and N_2O reducers

3.1.2.1 Ammonia oxidizers

Higher (1.6 - 4.4) AOA/AOB ratios were detected in the wheat straw amendments compared with the legume-grass mixture amendments (0.1 - 1.1) as shown in Figure 8. The influence of freezing and thawing resulted in an increase of AOA/AOB ratios in legume-grass mixtures amended samples from 0.4 to 1.1 compared with non-frozen

control soils which were around 0.2 - 0.6 at T0, T1 and T2 and decreased to 0.1 at T3. In the wheat straw amendments, AOA/AOB ratios increased in both freeze-thaw treated and non-frozen control samples. Specifically the freeze-thaw sample went from 1.8 to 3.9 and the control went from 1.8 to 4.4. In the last thawing period the ratios at T3 in freeze-thaw treated samples decreased from 3.9 to 1.6.

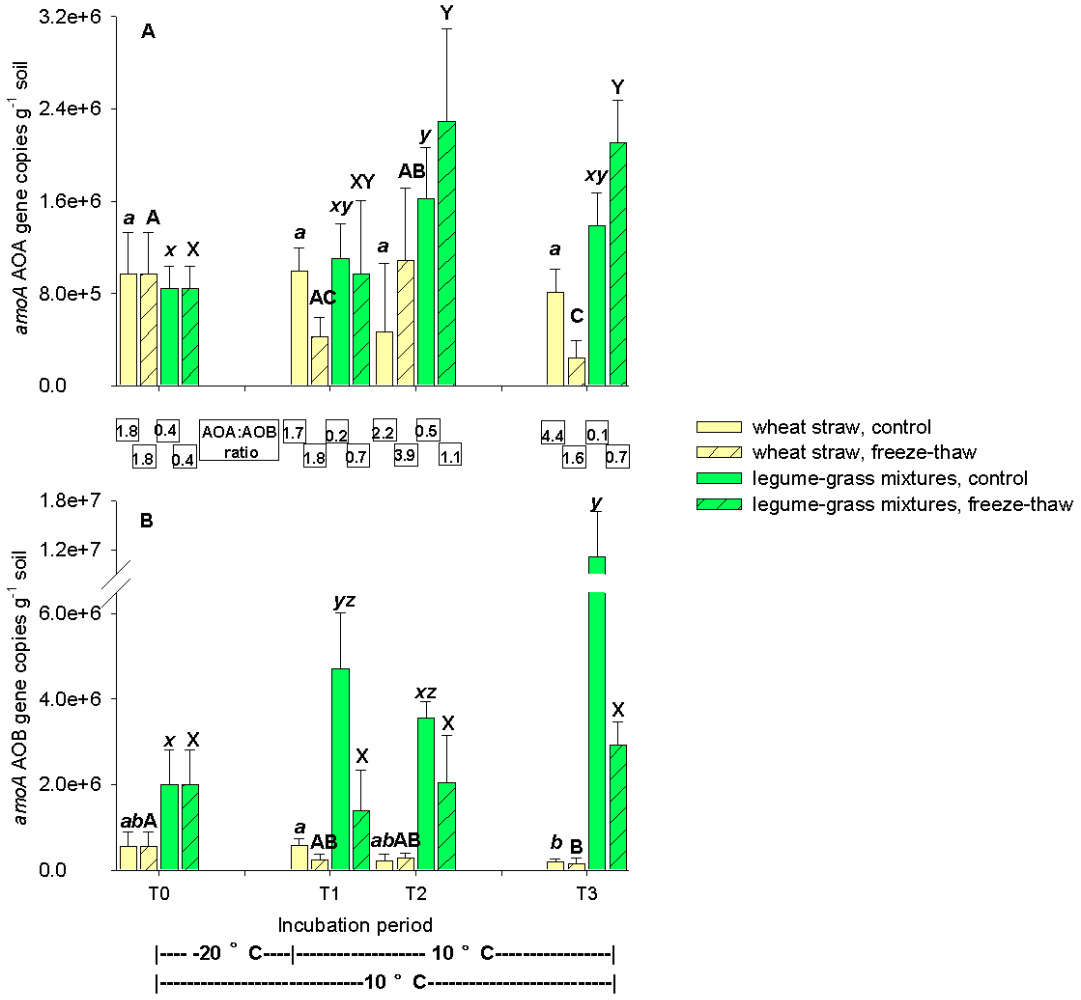

Figure 8 Abundance of ammonia oxidation related genes *amoA* AOA (A) and *amoA* AOB (B) in E1. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n = 4). Ratios of AOA to AOB *amoA* gene copies are shown in boxes between Figure 8 A and B. Different letters display significant differences at the different time points.

Significantly higher abundances of genes involved in ammonia oxidation were determined in the soil amended with legume-grass mixtures (AOA 0.8 - 1.6×10^6 and AOB 0.2 - 1.1×10^7 copies g^{-1} soil) than in the wheat straw amendment (AOA 4.7 - 9.7×10^5 and AOB 1.8 - 5.8×10^5 copies g^{-1} soil) (see Figure 8).

In the legume-grass mixtures amended soil samples, freeze-thaw cycles significantly reduced the increasing rate of *amoA* AOB gene copies compared with the control samples at T3 (freeze-thaw, 1.5 times; control, 5.5 times). No difference on *amoA* AOA gene abundance was detected as shown in Table 7. Similar *amoA* AOA gene copy numbers were detected between freeze-thaw treated and control samples (1.1 - 2.3×10^6 copies g^{-1} soil) at T1, T2 and T3. Furthermore, sampling time points showed a significant increase in both *amoA* AOA and *amoA* AOB gene copy numbers (see Table 7). In detail, for the *amoA* AOA gene, significant differences were detected between T0 (8.5×10^5 copies g^{-1} soil) and T2 (freeze-thaw, 2.3×10^6 copies g^{-1} soil; control, 1.6×10^6 copies g^{-1} soil). Those differences were also found between T0 and T3 (2.1×10^6 copies g^{-1} soil) in freeze-thaw treated samples. However, for *amoA* AOB genes no difference was found in samples which had been subjected to freezing and thawing; whereas a significant increase was detected from T0 to T2. The copy number decreased until 1.4×10^6 copies g^{-1} soil at T3 in the non-frozen control soil.

There was no significant influence of freezing and thawing in the soils amended with wheat straw on *amoA* AOA gene copy numbers. A major change was detected between freeze-thaw treated and non-frozen control samples in different sampling days during the thawing period at T1, T2 and T3. Briefly, higher *amoA* AOA gene copies (1.1×10^6 copies g^{-1} soil) were detected in samples which had been subjected to freezing and thawing at T2 compared with non-frozen control samples (4.7×10^5 copies g^{-1} soil); whereas, higher *amoA* AOA gene copies (8.1 - 9.9×10^5 copies g^{-1} soil) were detected in non-frozen control samples compared with samples which had been subjected to freezing and thawing (2.4 - 4.3×10^5 copies g^{-1} soil) at T1 and T3.

Table 7 Statistical evaluations of ammonia oxidation related genes using ANOVA in E1. The *p* values describe the impacts and interactions of sampling time points, soil treatments and amendments on ammonia oxidation related functional genes (*amoA* AOA and *amoA* AOB). Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values	
	amoA (AOA)	*amoA* (AOB)
Total		
Time (T0, T1, T2,T3)	0.89	0.03 *
Treatment (freeze-thaw, control)	0.91	0.68
Amendment (legume-grass mixtures, wheat straw)	0.00 *	0.00 *
Time × treatment	0.00 *	0.01 *
Time × amendment	0.00 *	0.01 *
Treatment × amendment	0.86	0.02 *
Time × treatment × amendment	0.02 *	0.03 *
Legume-grass mixtures amendment		
Time (T0, T1, T2,T3)	0.01 *	0.01 *
Treatment (freeze-thaw, control)	0.77	0.00 *
Time × treatment	0.30	0.07
Wheat straw amendment		
Time (T0, T1, T2,T3)	0.14	0.03 *
Treatment (freeze-thaw, control)	0.97	0.17
Time × treatment	0.01 *	0.03 *

Furthermore, a significant influence of sampling time and the interaction between freeze-thaw cycles and sampling time points was detected on *amoA* AOB gene copy numbers in the wheat straw amendment (see Table 7).

In detail, *amoA* AOB gene abundances were found to increase from T0 (5.5×10^5 copies g^{-1} soil) to T1 (5.8×10^5 copies g^{-1} soil) in non-frozen control samples; the numbers decreased from T0 to T1 (2.4×10^5 copies g^{-1} soil) in soil samples which had been subjected to freezing and thawing. Additionally a significant decrease was also shown from T1 to T3 (freeze-thaw, 1.6×10^5 copies g^{-1} soil; control, 1.8×10^5 copies g^{-1} soil).

3.1.2.2 Nitrite and N_2O reducers

Significantly higher gene copy numbers of nitrite and N_2O reducers were detected in soil samples amended with legume-grass mixtures (*nirK*, 2.0 - 2.5×10^8 copies g^{-1} soil; *nirS*, 0.4 - 1.6×10^7 copies g^{-1} soil; *nosZ*, 2.1 - 4.1×10^7 copies g^{-1} soil) than in wheat straw amended samples (*nirK*, 0.4 - 1.5×10^8 copies g^{-1} soil; *nirS*, 1.4 - 4.6×10^6 copies g^{-1} soil; *nosZ*, 1.4 - 8.0×10^6 copies g^{-1} soil) during the experiment independent of the time point and the treatment (see Table 8).

During the incubation period, independent of the freezing and thawing treatment, higher *nirK* gene copy numbers (0.4 - 2.5×10^8 copies g^{-1} soil) than *nirS* gene copy numbers (1.4×10^6 - 4.1×10^7 copies g^{-1} soil) were detected in both wheat straw and legume-grass mixtures amended samples as shown in Figure 9 A, B.

In the soil samples that were amended with legume-grass mixtures, freeze-thaw cycles did not significantly change the abundance of the measured genes involved in denitrification compared to the control samples (see Table 8). Similar gene copy numbers were detected between samples which had been subjected to freezing and thawing and non-frozen control soil samples for n*irK* gene in T3 (around 2.0×10^8 copies g^{-1} soil) and *nirS* gene in T2 (1.6×10^7 copies g^{-1} soil) and T3 (9.0×10^6 copies g^{-1} soil). During the experiment a significant increase of *nirS* genes from T0 (3.6×10^6 copies g^{-1} soil) to T3 (freeze-thaw, 1.7×10^7 copies g^{-1} soil; control, 1.6×10^7 copies g^{-1} soil) was observed.

Similar results were observed for soils amended with wheat straw (see Table 8). Similar gene copy numbers were detected between samples which had been subjected to freezing and thawing and non-frozen control soil samples for n*irK*, *nirS* and *nosZ* gene at T3 (*nirK*, around 4.4×10^7 copies g^{-1} soil; *nirS*, around 4.4×10^6 copies g^{-1} soil; *nosZ*, around 5.6×10^6 copies g^{-1} soil, Figure 9 A, B, C).

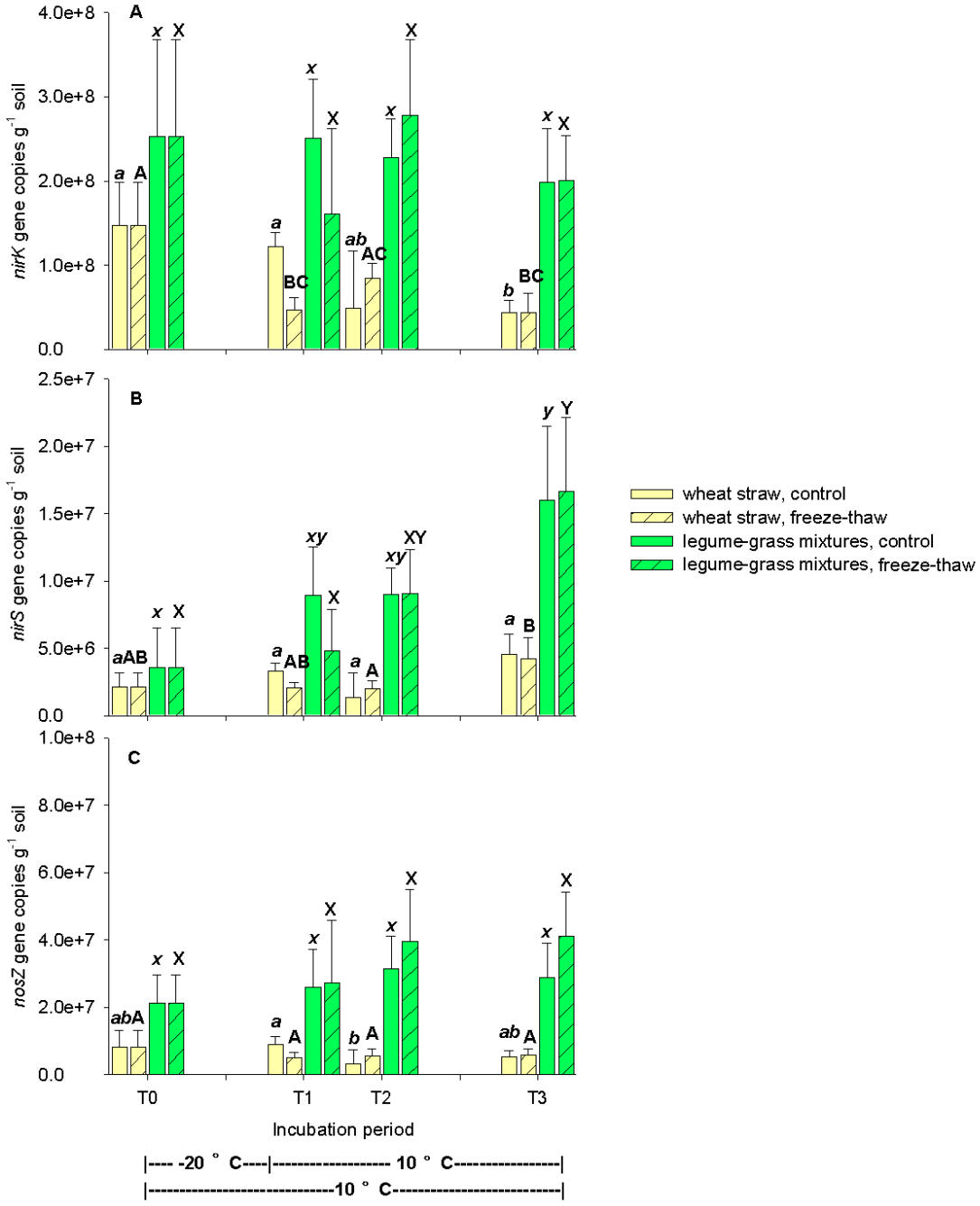

Figure 9 Abundance of nitrite and N_2O reducers related genes *nirK* (A), *nirS* (B) and *nosZ* (C) in E1. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n = 4). Different letters display significant differences at the different time points.

In the samples amended with the legume grass mixture significant differences of sampling time points were detected on *nirK* and *nosZ* genes in soil samples amended with wheat straw (see Table 8). In brief, the *nirK* gene copies showed a significant decrease from T0 (1.5×10^8 copies g^{-1} soil) to T3 (4.4×10^7 copies g^{-1} soil) in both freeze-thaw treated and control samples. For the *nosZ* gene, no difference was detected in freeze-thaw treated samples during the thawing period (T1, T2 and T3). However, there was a significant decrease from T1 (9.0×10^6 copies g^{-1} soil) to T2 (3.1×10^6 copies g^{-1} soil) in non-frozen control samples.

Table 8 Statistical evaluations of nitrite and N_2O reducers related genes using ANOVA in E1. The *p* values describe the impacts and interactions of sampling time point, soil treatments and amendments on nitrite and N_2O reducers related functional genes (*nirK*, *nirS* and *nosZ*). Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values		
	nirK	*nirS*	*nosZ*
Total			
Time (T0, T1, T2,T3)	0.03 *	0.00 *	0.21
Treatment (freeze-thaw, control)	0.68	0.95	0.37
Amendment (legume-grass mixtures, wheat straw)	0.00 *	0.00 *	0.00 *
Time × treatment	0.01 *	0.10	0.03 *
Time × amendment	0.02 *	0.01 *	0.01 *
Treatment × amendment	0.26	0.36	0.54
Time × treatment × amendment	0.15	0.41	0.11
Legume-grass mixtures amendment			
Time (T0, T1, T2,T3)	0.42	0.00 *	0.19
Treatment (freeze-thaw, control)	0.44	0.56	0.77
Time × treatment	0.30	0.76	0.73
Wheat straw amendment			
Time (T0, T1, T2,T3)	0.02 *	0.01 *	0.03 *
Treatment (freeze-thaw, control)	0.39	0.48	0.40
Time × treatment	0.02 *	0.05	0.04 *

3.2 Influence of freeze-thaw cycles on ammonia oxidizers and selected denitrifiers in soils amended with litter differing in the amount of carbon (E2)

3.2.1 Soil chemical analyses

Total C input through the wheat straw, corresponding to 3.5×10^4 µg C g^{-1} soil, was twelve times higher than the legume-grass mixtures, corresponding to 2.8×10^3 µg C g^{-1} soil. Soils amended with wheat straw showed 3.0 - 4.0 times higher WEOC content at 97.1 - 126.8 µg g^{-1} soil compared to the legume-grass mixtures amended samples at 30.7 - 48.3 µg g^{-1} soil independent from the freezing and thawing treatments and time points investigated (see Figure 10 A). Although similar N contents, corresponding to 2.7×10^2 µg N g^{-1} soil, were added to the soil samples before pre-incubation, significantly higher TNb , NH_4^+ and NO_3^- contents at 5.6 - 13.5µg TNb, 2.4 - 8.8 µg NH_4^+ and 0.7 - 2.0 µg NO_3^- per gram of soil, respectively, were detected in the soil samples amended with legume-grass mixtures compared with wheat straw at 5.2 - 6.2 µg TNb, up to 0.03 µg NH_4^+ and 0.06 - 0.14 µg NO_3^- per gram of soil, respectively, during the experiment (see Figure 10). Specifically, the $NH4^+$ contents from legume-grass mixture amended soil samples were 90 - 1485 times higher when compared with the wheat straw amendment. NO_3^- contents were 7 - 28 times higher in legume-grass mixture amendment than in wheat straw amendment.

In legume-grass mixtures amended soil samples, the freeze-thaw effect significantly influenced the TNb and NH_4^+ content. In detail, samples which had been subjected to freezing and thawing revealed significantly higher amounts of TNb (7.5 - 13.5 µg g^{-1} soil) and NH_4^+ content (4.2 - 8.8 µg g^{-1} soil) compared to the non-frozen control samples (TNb, 5.9 - 8.0 µg g^{-1}; NH_4^+, 2.4 - 3.3 µg g^{-1} soil). Moreover, sampling time points and the interaction between freeze-thaw stress and time revealed significant effects on WEOC, TNb and NH_4^+ contents as shown in Table 9. Briefly, the WEOC

content showed significant differences between T1 (31.0 µg g^{-1} soil) and T2 (48.3 µg g^{-1} soil) in non-frozen control samples, whereas, no difference was found in freeze-thaw treated samples.

For TNb content, a significant decrease was detected from T1 (13.5 µg g^{-1} soil) to T3 (7.5 µg g^{-1} soil) in samples which had been subjected to freezing and thawing, while an increase was measured from T1 (5.9 µg g^{-1} soil) to T3 (7.1 µg g^{-1} soil) in non-frozen control samples.

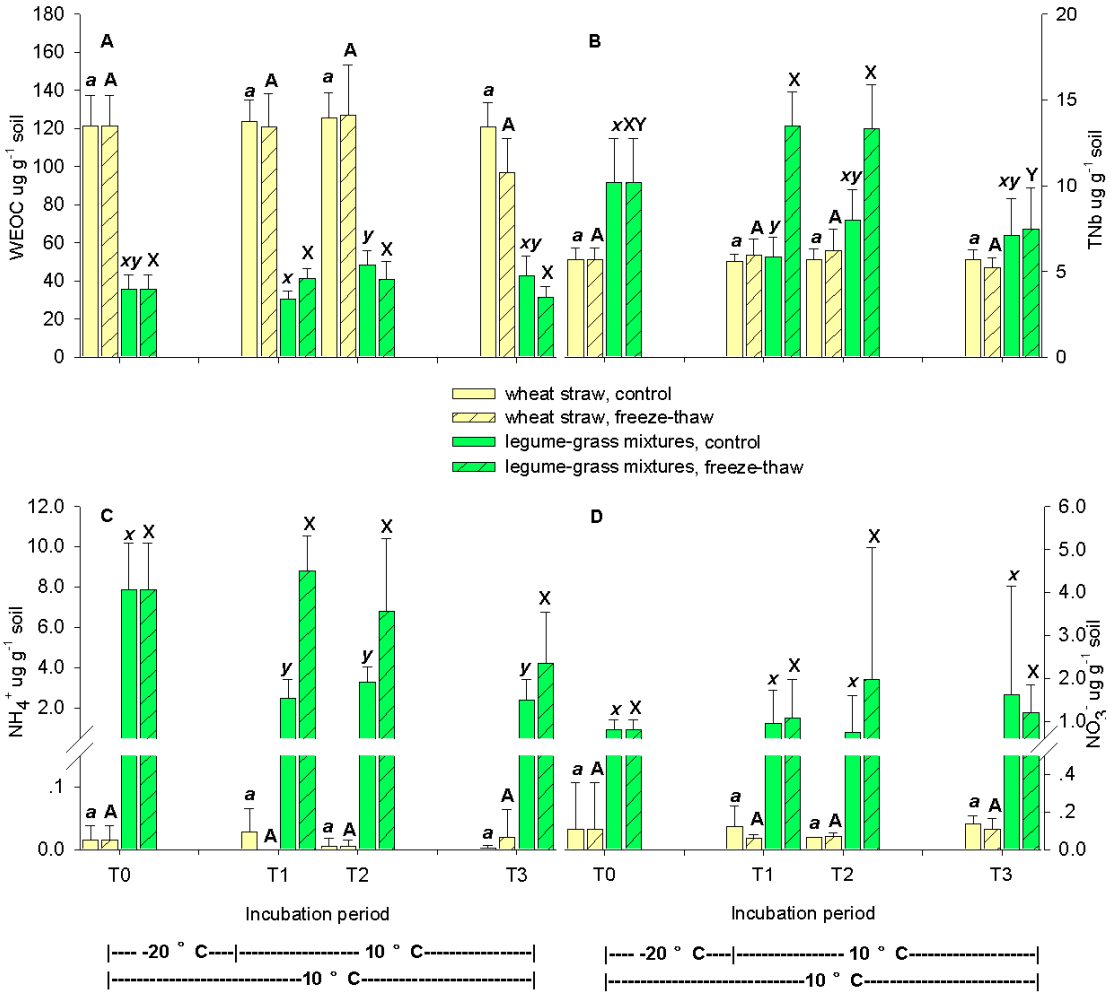

Figure 10 Amounts of WEOC (A), TNb (B), NH_4^+ (C) and NO_3^- (D) in E2. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n = 5). Different letters display significant differences at the different time points.

However, TNb content was increased in samples which had been subjected to freezing and thawing, and decreased in non-frozen control samples from T0 (10.2 μg g^{-1} soil) to T1. Significant decrease of NH_4^+ concentration was shown from T0 (7.9 μg g^{-1} soil) to T3 (2.4 μg g^{-1} soil) in non-frozen control soil. However, no difference in samples which had been subjected to freezing and thawing was observed.

There was no significant difference in freeze-thaw cycles, sampling time points and the interaction between each soil parameter (WEOC, TNb, NH_4^+ and NO_3^-) in soil samples amended with wheat straw (see Table 9).

Table 9 Statistical evaluations of the chemical parameters of the soil using ANOVA in E2. The *p* values describe the impacts and interactions of sampling time point, soil treatments and amendments on soil WEOC, TNb, NH_4^+ and NO_3^-. Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values			
	WEOC	TNb	NH_4^+	NO_3^-
Total				
Time (T0, T1, T2,T3)	0.04 *	0.00 *	0.00 *	0.80
Treatment (freeze-thaw, control)	0.17	0.00 *	0.00 *	0.65
Amendment (legume-grass mixtures, wheat straw)	0.00 *	0.00 *	0.00 *	0.00 *
Time × treatment	0.07	0.00 *	0.01 *	0.65
Time × amendment	0.29	0.03 *	0.00 *	0.78
Treatment × amendment	0.47	0.00 *	0.00 *	0.59
Time × treatment × amendment	0.49	0.00 *	0.01 *	0.68
Legume-grass mixtures amendment				
Time (T0, T1, T2,T3)	0.03 *	0.01 *	0.00 *	0.788
Treatment (freeze-thaw, control)	0.42	0.00 *	0.00 *	0.621
Time × treatment	0.02 *	0.00 *	0.02 *	0.660
Wheat straw amendment				
Time (T0, T1, T2,T3)	0.15	0.50	0.76	0.81
Treatment (freeze-thaw, control)	0.25	0.68	0.73	0.61
Time × treatment	0.33	0.48	0.23	0.93

3.2.2 Abundances of ammonia oxidizers and nitrite and N_2O reducers

3.2.2.1 Ammonia oxidizers

Although similar N content was added to both amended soils, higher AOA/AOB ratios of 2.7 - 4.9 were detected in wheat straw compared to the legume-grass mixture amendment at 1.2 - 1.9. This is shown in Figure 11. Furthermore, significant higher gene copy numbers of archaeal *amoA* AOA genes (1.6 - 7.8×10^6 *amoA* copies g^{-1} soil, Figure 11 A) were found in all samples compared to the bacterial counterpart *amoA* AOB (5.2×10^5 - 3.3×10^6 *amoA* copies g^{-1} soil, Figure 11 B). However, the influence of freezing and thawing resulted in an increase of AOA/AOB ratios in wheat straw amended samples. They ranged from 2.7 to 4.9 compared with the non-frozen control soil samples which increased from 2.7 to 3.7 and then decreased to between 2.9 and 3.4. In legume-grass mixtures amended samples, AOA/AOB ratios increased in both freeze-thaw treated and control samples. The freeze-thaw sample went from 1.6 to 1.7 and the control went from 1.6 to 1.9. However, by T3, the AOA/AOB ratios had decreased in freeze-thaw treated soil from 1.7 to 1.5 and in non-frozen control samples from 1.9 to 1.3.

Significantly higher abundances of *amoA* AOB gene were found in the soil amended with legume-grass mixtures (0.9 - 3.3×10^6 copies g^{-1} soil) compared to the wheat straw amendment (0.5 - 2.3×10^5 copies g^{-1} soil). This is shown in Figure 11 B. However, there was no difference in *amoA* AOA gene abundances between amendments ranging between 1.6 - 7.8×10^6 copies g^{-1} soil.

In soil samples amended with the legume-grass mixtures, freezing and thawing significantly reduced the growth rate of *amoA* AOB gene copies compared with the control samples. The freeze-thaw sample increased by 1.4 times whereas the control increased by 3.6 times. Significant effects of sampling time points are shown in Table 10. There was significant interaction between sampling times and treatments on the

amoA AOB gene. In detail, there was a significant increase in *amoA* AOB gene abundances found from T0 (1.1×10^6 copies g^{-1} soil) to T3 (3.3×10^6 copies g^{-1} soil) in the non-frozen control samples. There was no difference in freeze-thaw treated soil during the whole incubation time. Furthermore, no difference in *amoA* AOA gene copies was detected between samples which had been subjected to freezing and thawing and non-frozen control samples. There was also no effect of sampling time points on the *amoA* AOA genes, either in samples which had been subjected to freezing and thawing or in the non-frozen control samples (see Table 10).

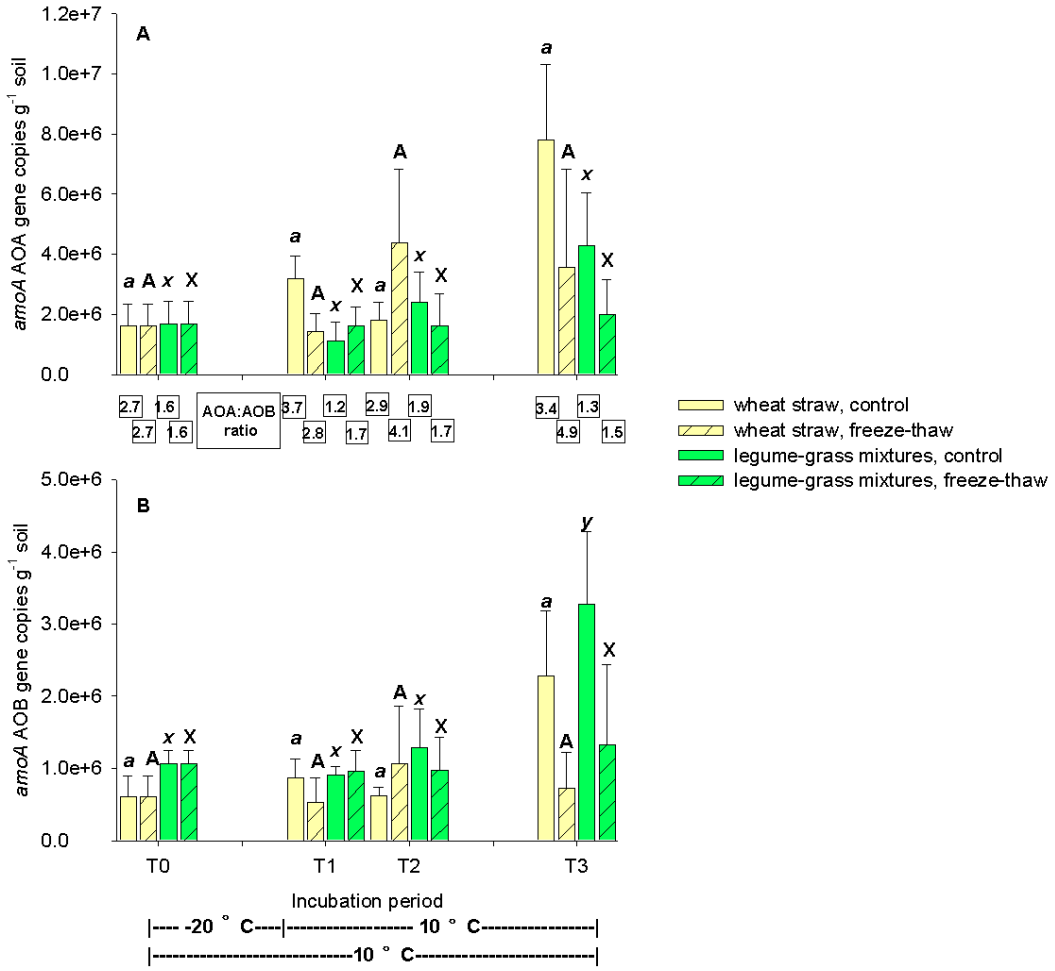

Figure 11 Abundance of ammonia oxidizers related genes *amoA* AOA (A) and *amoA* AOB (B) in E2. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). Error bars indicated standard deviation of mean (n=5). Ratios of AOA to AOB *amoA* gene copies are shown in boxes between Figure 11 A and B. Different letters display significant differences at the different time points.

Like in the legume-grass mixture amendments, no significant influence of freeze-thaw cycles were detected on *amoA* AOA gene in wheat straw amended soil samples (see Table 10). There was also no effect of sampling times on *amoA* AOB gene. However, significant differences between the freeze-thaw treated and the control treatments were detected on *amoA* AOB gene in the wheat straw amendment. In brief, a higher number of *amoA* AOB gene copies were detected in samples which had been subjected to freezing and thawing at T2 (1.1×10^6 copies g^{-1} soil) compared with the non-frozen control samples (6.1×10^5 copies g^{-1} soil). At T1 and T3, higher *amoA* AOB gene copies (0.9 - 2.3×10^6 copies g^{-1} soil) were detected in the non-frozen control samples compared with the freeze-thaw treated samples (5.2 - 7.2×10^5 copies g^{-1} soil).

Table 10 Statistical evaluations of ammonia oxidizers related genes using ANOVA in E2. The *p* values describe the impacts and interactions of sampling time point, soil treatments and amendments on ammonia oxidizers related functional genes (*amoA* AOA and *amoA* AOB). Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values	
	amoA (AOA)	*amoA* (AOB)
Total		
Time (T0, T1, T2,T3)	0.13	0.03 *
Treatment (freeze-thaw, control)	0.06	0.00 *
Amendment (legume-grass mixtures, wheat straw)	0.20	0.00 *
Time × treatment	0.08	0.01 *
Time × amendment	0.68	0.96
Treatment × amendment	0.39	0.53
Time × treatment × amendment	0.11	0.62
Legume-grass mixtures amendment		
Time (T0, T1, T2,T3)	0.14	0.01 *
Treatment (freeze-thaw, control)	0.42	0.02 *
Time × treatment	0.16	0.17 *
Wheat straw amendment		
Time (T0, T1, T2,T3)	0.54	0.42
Treatment (freeze-thaw, control)	0.08	0.04 *
Time × treatment	0.08	0.15

3.2.2.1 Nitrite and N_2O reducers

Significantly higher numbers of gene copies of *nirK* were found in the soil samples amended with wheat straw (0.7 - 1.3×10^8 copies g^{-1} soil) in comparison to the legume-grass mixtures amended samples (2.4 - 6.6×10^7 copies g^{-1} soil). This is shown in Figure 12 A. Like the *nirK* gene, a significantly higher numbers of gene copies of *nosZ* genes were also found in the soil amended with wheat straw (1.3 - 6.2×10^7 copies g^{-1} soil) than in the legume-grass mixtures amended samples (0.5 - 2.3×10^7 copies g^{-1} soil, Figure 12 C). However, no significant differences of amendments on *nirS* gene were shown as both were around 1.1 - 3.1×10^6 copies g^{-1} soil (see Figure 12 B).

For genes involved in NO_2^- reduction, higher *nirK* gene copy numbers (2.4×10^7 - 1.3×10^8 copies g^{-1} soil) compared with *nirS* gene (1.1 - 4.4×10^6 copies g^{-1} soil) were detected independent of amendments and freeze-thaw and control treatments.

In the legume-grass mixtures added samples, no significant influence of freezing and thawing was visible on each denitrification related genes (*nirK*, 3.4 - 6.6×10^7 copies g^{-1} soil; *nirS*, 1.1 - 3.1×10^6 copies g^{-1} soil; *nosZ*, 0.8 - 2.3×10^7 copies g^{-1} soil) compared to non-frozen control samples (*nirK*, 2.4 - 5.9×10^7 copies g^{-1} soil; *nirS*, 1.2 - 1.6×10^6 copies g^{-1} soil; *nosZ*, 0.5 - 1.2×10^7 copies g^{-1} soil) as shown in Table 11.

However, sampling time points showed a significant decrease of the *nirS* gene copy numbers from T0 (4.4×10^6 copies g^{-1} soil) to T2 (freeze-thaw, 1.1×10^6 copies g^{-1} soil; control, 1.6×10^6 copies g^{-1} soil). At T3, these subsequently increased in samples which had been subjected to freezing and thawing (1.7×10^6 copies g^{-1} soil) and decreased in non-frozen control samples (1.2×10^6 copies g^{-1} soil).

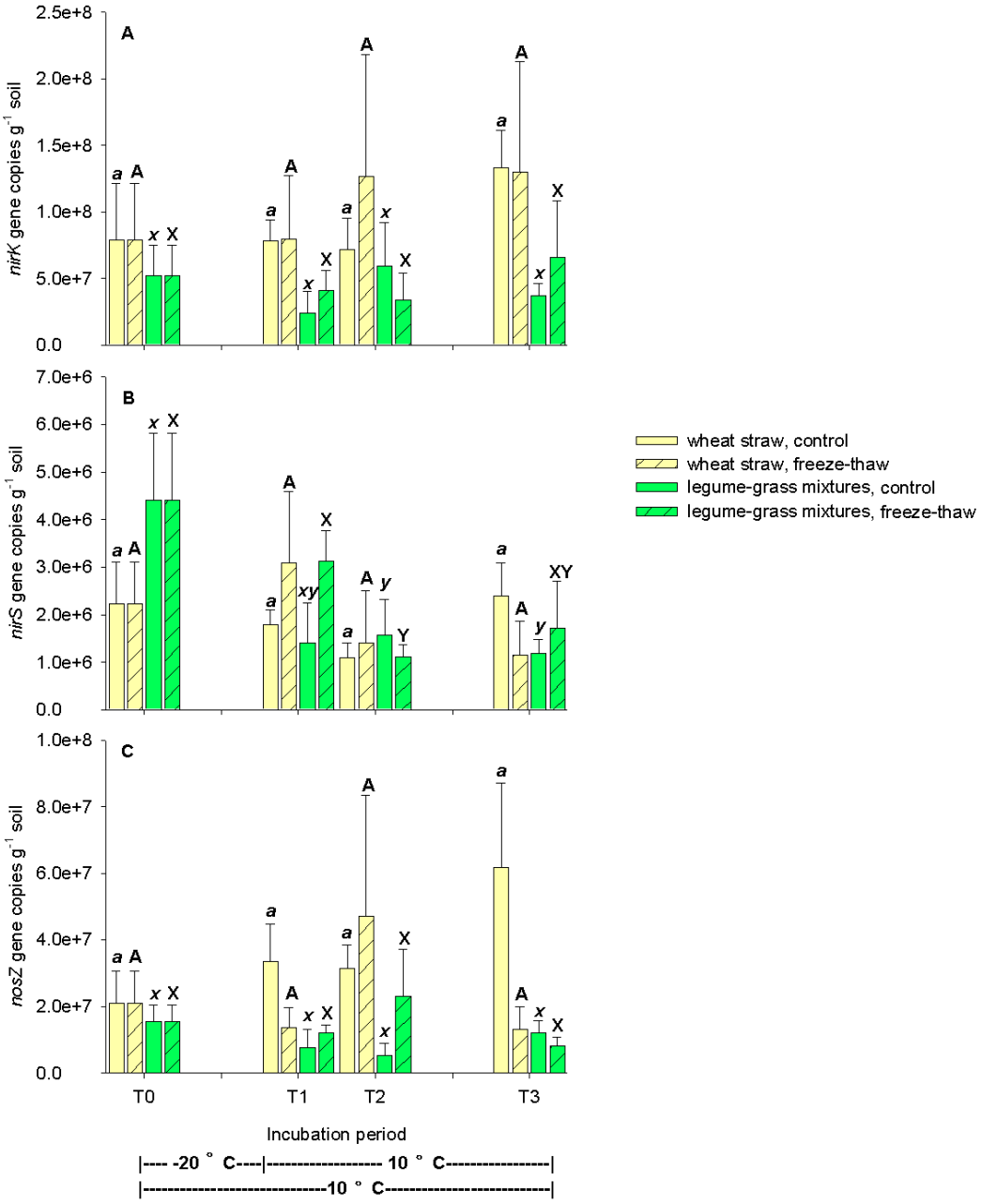

Figure 12 Abundance of nitrite and N_2O reducers related genes *nirK* (A), *nirS* (B) and *nosZ* (C) in E2. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n=5). Different letters display significant differences at the different time points.

There was no significant difference on sampling time points in soil samples amended with wheat straw during the incubated times (Table 11). Freeze-thaw cycles also did not result in significant effects on the *nirK* and *nirS* gene copy numbers. Specifically, similar gene copy numbers were detected between freeze-thaw treated and control soil samples for the *nirK* gene at T1 (around 7.9×10^7 copies g^{-1} soil) and T3 (around 1.3×10^8 copies g^{-1} soil) and for the *nirS* gene at T2 (around 1.2×10^6 copies g^{-1} soil). However, the *nosZ* gene was detected in significantly higher copy numbers in the non-frozen control soils (2.0 - 6.1×10^7 copies g^{-1} soil) than in samples which had been subjected to freezing and thawing (1.3 - 4.3×10^7 copies g^{-1} soil).

Table 11 Statistical evaluations of nitrite and N_2O reducers related genes using ANOVA in E2. The *p* values describe the impacts and interactions of sampling time point, soil treatments and amendments on nitrite and N_2O reducers related functional genes (*nirK*, *nirS* and *nosZ*). Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	ANOVA *p* values		
	nirK	*nirS*	*nosZ*
Total			
Time (T0, T1, T2,T3)	0.42	0.00 *	0.58
Treatment (freeze-thaw. control)	0.62	0.99	0.37
Amendment (legume-grass mixtures, wheat straw)	0.01 *	0.32	0.00 *
Time × treatment	0.41	0.07	0.37
Time × amendment	0.81	0.32	0.51
Treatment × amendment	0.57	0.09	0.03 *
Time × treatment × amendment	0.30	0.49	0.52
Legume-grass mixtures amendment			
Time (T0, T1, T2,T3)	0.43	0.00 *	0.34
Treatment (freeze-thaw. control)	0.96	0.16	0.39
Time × treatment	0.16	0.09	0.50
Wheat straw amendment			
Time (T0, T1, T2,T3)	0.88	0.15	0.94
Treatment (freeze-thaw. control)	0.42	0.30	0.02 *
Time × treatment	0.92	0.29	0.39

3.3 Influence of freeze-thaw cycles on ammonia oxidizers and selected denitrifiers in soils amended with litter and copper (E3)

3.3.1 Soil chemical analyses

In this experiment all the samples were amended with legume-grass mixtures (corresponding to 1.6×10^3 μg N g^{-1} soil and 1.7×10^4 μg C g^{-1} soil). The amounts were as same as in E1. Due to extra $CuCl_2$ (150 mg kg^{-1} soil) input, significant higher Cu^{2+} concentrations were detected in Cu^{2+} contaminated soils (3.2 - 20.4 μg g^{-1} soil) than in non Cu^{2+} contaminated soils (0.14 - 0.58 μg g^{-1} soil). This is shown in Figure 13 A.

As demonstrated in Table 12, significant influences of Cu^{2+} contamination were detected on the WEOC, NH_4^+ and NO_3^- contents when compared with non Cu^{2+} contaminated soil samples, independent of the freeze-thaw cycles. There were also significant interactions between the Cu^{2+} contamination and sampling time points. In detail, the WEOC contents from non Cu^{2+} contaminated soils were 1.3 times higher than Cu^{2+} contaminated samples at T0. In contrast, the WEOC content was 1.6 times higher in the Cu^{2+} contaminated samples than in the non Cu^{2+} contaminated soil at T3 (see Figure 13 B). NH_4^+ concentrations were 1.4 times higher in non Cu^{2+} contaminated soil samples than in Cu^{2+} contaminated samples at T0. Those differences were in contrast to the results at T3 where higher content in the Cu^{2+} contaminated samples was detected (see Figure 13 D). Although NO_3^- concentrations were 3.0 times higher in the non Cu^{2+} contaminated soil samples than in the Cu^{2+} contaminated samples at T0, 1.5 times lower content was found in the non Cu^{2+} contaminated samples compared with the Cu^{2+} contaminated soil samples at T1 in non-frozen samples (see Figure 13 E). No significant difference was shown on the TNb content between the Cu^{2+} contaminated (131 - 319 μg g^{-1} soil) and the non Cu^{2+} contaminated soil samples (130 - 399 μg g^{-1} soil, Figure 13 C).

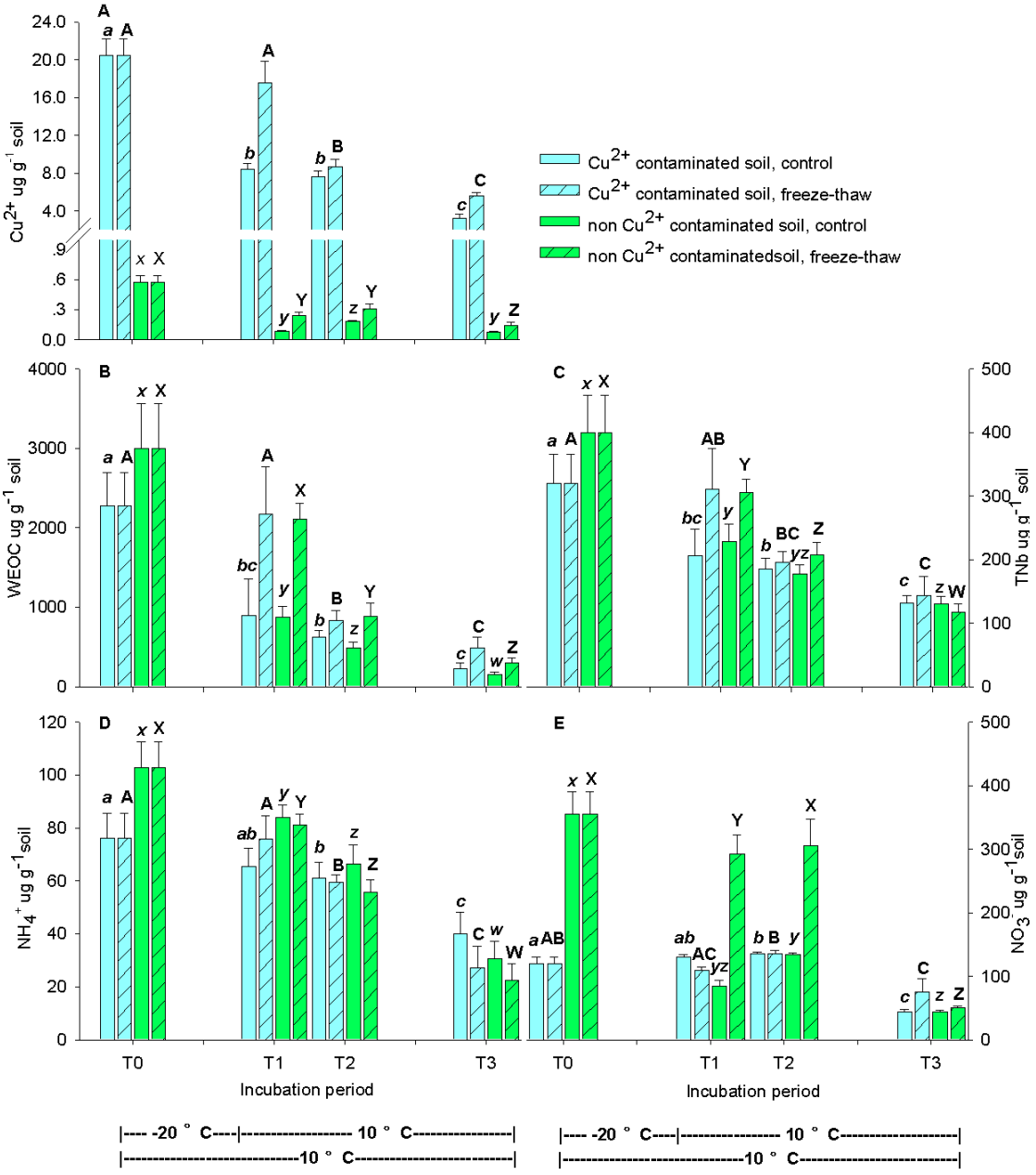

Figure 13 Amounts of soil Cu^{2+} content (A), WEOC (B), TNb (C), NH_4^+ (D) and NO_3^- (E) in E3. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n = 5). Different letters display significant differences at the different time points.

In the Cu^{2+} contaminated soil samples, significant higher Cu^{2+} (5.6 - 17.6 µg g^{-1} soil), WEOC (487.6 - 2170.2 µg g^{-1} soil) and TNb (143.6 - 311.4 µg g^{-1} soil) contents were detected in freeze-thaw treated soils than in non-frozen control samples (Cu^{2+}, 3.2 - 8.4 µg g^{-1} soil; WEOC, 229.4 - 894.5 µg g^{-1} soil; TNb, 131.0 - 206.0 µg g^{-1} soil). However, no significant effect of freeze-thaw cycles was found on soil NH_4^+ and NO_3^- contents (see Table 12). A significant effect of sampling time points and the interaction between freeze-thaw stress and time were detected on soil Cu^{2+}, WEOC, TNb, NH_4^+ and NO_3^- contents. In detail, the Cu^{2+} content described a significant decrease from T0 (20.4 µg g^{-1} soil) to T3 (freeze-thaw, 5.6 µg g^{-1} soil; control, 3.2 µg g^{-1} soil). No significant different of the Cu^{2+} content was detected between T0 and T1 in the freeze-thaw treated samples, while in the non-frozen control samples, a significantly higher Cu^{2+} content was shown at T0 than at T1.

Like the Cu^{2+} content, significant decreases in WEOC and TNb contents were detected from T0 (WEOC, 2273.0 µg g^{-1} soil; TNb, 319.5 µg g^{-1} soil) to T3 (freeze-thaw, 487.6 µg WEOC and 143.6 µg TNb per gram of soil; control, 229.4 µg WEOC and 131.0 µg TNb per gram of soil). Significantly higher WEOC and TNb content was also shown at T0 than T1 in non-frozen control samples. No significant difference was found in the samples which had been subjected to freezing and thawing between T0 and T1. A significant decrease in the NH_4^+ contents was also detected from T0 (76.0 µg g^{-1} soil) to T3 (freeze-thaw, 27.2 µg g^{-1} soil; control, 40.1 µg g^{-1} soil). However, there was no difference between T0 and T1 in both freeze-thaw treated and control samples. For NO_3^- contents, a significant increase was detected from T0 (120.0 µg g^{-1} soil) to T2 (freeze-thaw, 135.6 µg g^{-1} soil; control, 136.1 µg g^{-1} soil). It then decreased until it reached 76.0 µg g^{-1} soil in the freeze-thaw treated samples and 43.7 µg g^{-1} soil in the non-frozen control samples at T3.

Table 12 Statistical evaluations of the chemical parameters of the soil using ANOVA in E3. The *p* values describe the impacts and interactions of sampling time point, soil treatments and Cu^{2+} contamination on soil WEOC, TNb, NH_4^+, NO_3^- and concentration of soil Cu^{2+}. Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values				
	Cu^{2+}	WEOC	TNb	NH_4^+	NO_3^-
Total					
Time (T0, T1, T2,T3)	0.00 *	0.00 *	0.00 *	0.00 *	0.00 *
Treatment (frozen, non-frozen)	0.00 *	0.00 *	0.00 *	0.06	0.35
Cu^{2+} (contaminated, non contaminated)	0.00 *	0.09	0.02 *	0.00 *	0.00 *
Time × treatment	0.00 *	0.00 *	0.00 *	0.02 *	0.00 *
Time × Cu^{2+}	0.00 *	0.00 *	0.00 *	0.00 *	0.00 *
Treatment × Cu^{2+}	0.00 *	0.96	0.60	0.19	0.39
Time × treatment × Cu^{2+}	0.00 *	0.91	0.69	0.21	0.00 *
Cu^{2+} contamination					
Time (T0, T1, T2,T3)	0.00 *	0.00 *	0.00 *	0.00 *	0.00 *
Treatment (frozen, non-frozen)	0.00 *	0.00 *	0.01*	0.69	0.86
Time × treatment	0.00 *	0.00 *	0.01*	0.02 *	0.00 *
Non Cu^{2+} contamination					
Time (T0, T1, T2,T3)	0.00 *	0.00 *	0.00 *	0.00 *	0.00 *
Treatment (frozen, non-frozen)	0.00 *	0.00 *	0.03*	0.02 *	0.36
Time × treatment	0.00 *	0.00 *	0.03*	0.32	0.00 *

Significant freeze-thaw effects in non Cu^{2+} contaminated soil samples were detected on the Cu^{2+}, WEOC, TNb and NH_4^+ contents. Significantly higher Cu^{2+} (0.14 - 0.31 µg g^{-1} soil), WEOC (303.5 - 2104.4 µg g^{-1} soil) and TNb (117.8 - 306.1 µg g^{-1} soil) contents were found in freeze-thaw treated soil samples compared to control samples (Cu^{2+}, 0.08 - 0.18 µg g^{-1} soil; WEOC, 147.8 - 879.3 µg g^{-1} soil; TNb, 130.2 - 229.1 µg g^{-1} soil; Figure 13).

In contrast , significantly higher NH_4^+ content was detected in non-frozen control soil samples (30.6 - 83.8 µg g^{-1} soil) than in samples which had been subjected to freezing and thawing (22.5 - 81.1 µg g^{-1} soil, Figure 13 C). Significant effects of sampling time points were detected on soil Cu^{2+}, WEOC, TNb and NO_3^- (see Table 12). There was

also significant interaction between freeze-thaw stress and sampling times. In detail, the Cu^{2+} content showed significant decrease from T0 (0.58 μg g^{-1} soil) to T1 (freeze-thaw, 0.2 μg g^{-1} soil; control, 0.1 μg g^{-1} soil) and an increase to 0.3 μg g^{-1} soil (freeze-thaw treated samples) and 0.2 μg g^{-1} soil (non-frozen control samples) at T2. There was a finally decrease to 0.1 μg g^{-1} soil in both freeze-thaw treated and control samples at T3. A significant decrease in the WEOC content was shown from T0 (2994.9 μg g^{-1} soil) to T3 (freeze-thaw, 303.5 μg g^{-1} soil; control, 147.8 μg g^{-1} soil). However, there was no difference in the WEOC content detected between T0 and T1 in samples which had been subjected to freezing and thawing. For TNb and NH_4^+ contents, significant decreases were detected from T0 (TNb, 399.4 μg g^{-1} soil; NH_4^+, 102.8 μg g^{-1} soil) to T3 in both freeze-thaw treated (TNb, 117.8 μg g^{-1} soil; NH_4^+, 22.5 μg g^{-1} soil) and control samples (TNb, 130.2 μg g^{-1} soil; NH_4^+, 30.6 μg g^{-1} soil). Moreover, NO_3^- concentrations significantly decreased from T0 (355.6 μg g^{-1} soil) to T1 (freeze-thaw, 293.11 μg g^{-1} soil; control, 84.9 μg g^{-1} soil) and increased to 306.2 μg g^{-1} soil (freeze-thaw treated samples) and 134.9 μg g^{-1} soil (non-frozen control samples) at T2. There was a final decrease until 50.0 μg g^{-1} soil (freeze-thaw treated samples) and 43.8 μg g^{-1} soil (non-frozen control samples) at T3.

3.3.2 Abundance of ammonia oxidizers and nitrite and N_2O reducers

3.3.2.1 Ammonia oxidizers

Reflecting the AOA/AOB ratios of lower than 1 shown in Figure 14, higher *amoA* gene abundances were found in the ammonia oxidizing bacterial (AOB) than in the ammonia oxidizing archaeal (AOA) for both Cu^{2+} contaminated and non Cu^{2+} contaminated soil samples during the investigation period independent of the copper treatmeant. Although higher Cu^{2+} contamination was added to half of the samples, there was no significant difference of AOA/AOB ratios detected in either Cu^{2+} contaminated or non Cu^{2+} contaminated soil samples.

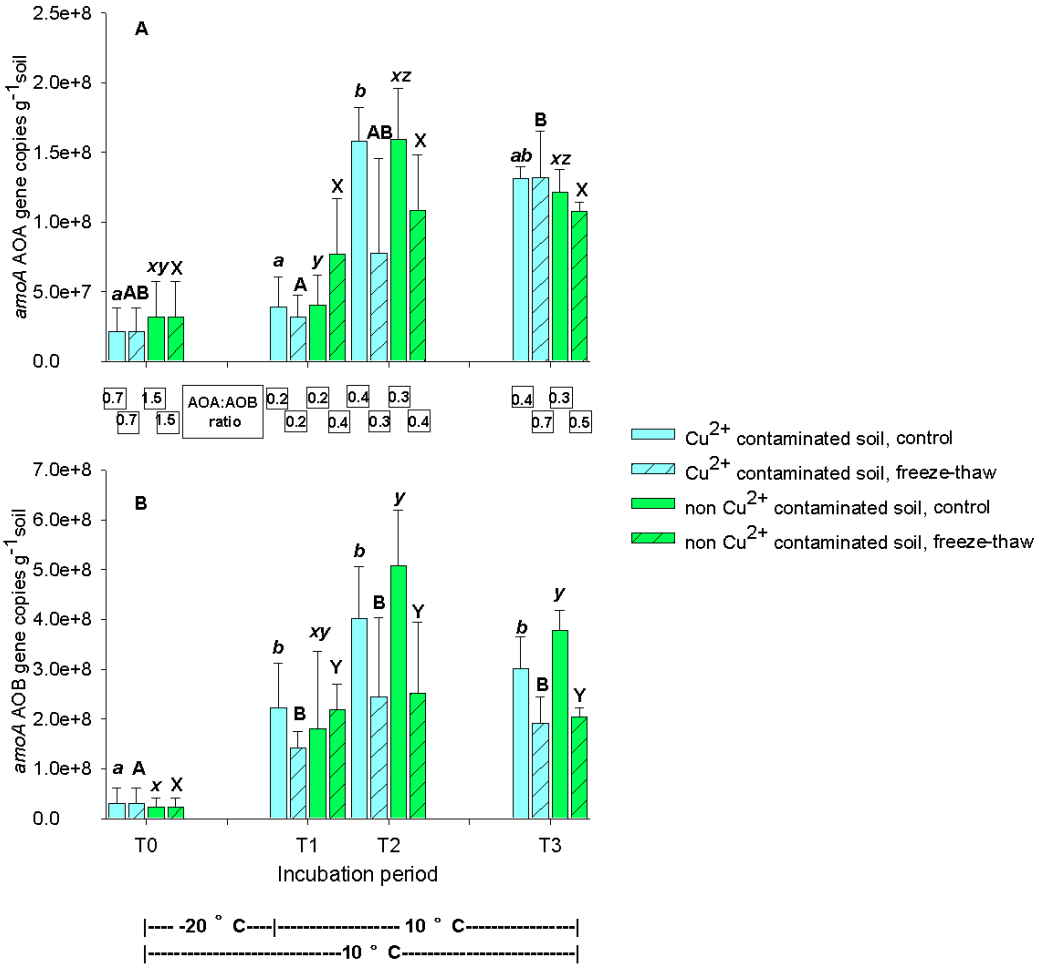

Figure 14 Abundance of ammonia oxidizers related genes *amoA* AOA (A) and *amoA* AOB (B) in E3. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n=5). Ratios of AOA to AOB *amoA* copies are shown in boxes between Figure 14 A and B. Different letters display significant differences at the different time points.

The influence of freezing and thawing resulted in a decrease of AOA/AOB ratios from 0.7 to 0.2 at T1 and then increasing from 0.2 to 0.7 at T3 in Cu^{2+} contaminated samples. In non Cu^{2+} contaminated soil, however, AOA/AOB ratios first decreased in both freeze-thaw treated (from 1.5 to 0.2) and control samples (from 1.5 to 0.4), and then increased (freeze-thaw, from 0.4 to 0.5; control, from 0.2 to 0.4). Significantly higher abundances of *amoA* AOB gene were detected in non-frozen control samples (2.2×10^7 - 5.1×10^8 *amoA* copies g^{-1} soil) compared with samples which had been subjected to freezing and thawing (2.2×10^7 - 2.5×10^8 *amoA* copies g^{-1} soil, Figure 14

B). However, there was no significant influence of freeze-thaw stress on the *amoA* AOA genes as shown in Table 13.

No significant influence of freezing and thawing on *amoA* AOA and *amoA* AOB gene copy numbers was detected in Cu^{2+} contaminated soil samples (see Table 13). However, sampling time points showed significant effects on both *amoA* AOA and AOB gene copy numbers. In brief, a significant increase of *amoA* AOA gene copy numbers was detected from T0 (2.2×10^7 *amoA* copies g^{-1} soil) to T3 (1.3×10^8 *amoA* copies g^{-1} soil) in both freeze-thaw treated and control samples.

Table 13 Statistical evaluations of ammonia oxidizers related genes using ANOVA in E3. The *p* values describe the impacts and interactions of sampling time point, soil treatments and Cu^{2+} contamination on ammonia oxidizers related functional genes (*amoA* AOA and *amoA* AOB). Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values	
	amoA (AOA)	*amoA* (AOB)
Total		
Time (T0, T1, T2,T3)	0.00 *	0.00 *
Treatment (freeze-thaw, control)	0.39	0.04 *
Cu^{2+} (contaminated, non contaminated)	0.11	0.99
Time × treatment	0.23	0.12
Time × Cu^{2+}	0.53	0.64
Treatment × Cu^{2+}	0.37	0.60
Time × treatment × Cu^{2+}	0.76	0.52
Cu^{2+} contamination		
Time (T0, T1, T2,T3)	0.00 *	0.00 *
Treatment (freeze-thaw, control)	0.24	0.05
Time × treatment	0.46	0.63
Non Cu^{2+} contamination		
Time (T0, T1, T2,T3)	0.00 *	0.00 *
Treatment (freeze-thaw, control)	0.97	0.32
Time × treatment	0.41	0.14

A significant increase of *amoA* AOA gene copy numbers was detected from T0 (3.0×10^7 *amoA* copies g^{-1} soil) to T2 (freeze-thaw, 2.4×10^8 *amoA* copies g^{-1} soil; control, 4.0×10^8 *amoA* copies g^{-1} soil). They then decreased until 1.9×10^8 *amoA* copies g^{-1} soil in the freeze-thaw treated samples and 3.0×10^8 *amoA* copies g^{-1} soil in the non- frozen control samples at T3.

There was no influence of freezing and thawing in non Cu^{2+} contaminated soil samples on either the *amoA* AOA or the AOB gene. However, sampling time points showed significant differences. Briefly, a significant increase of *amoA* AOA gene abundances was detected from T0 (3.2×10^7 *amoA* copies g^{-1} soil) to T3 (1.1×10^8 *amoA* copies g^{-1} soil) in samples which had been subjected to freezing and thawing. In non-frozen control samples, a significant increase was shown from T0 to T2 (1.6×10^8 *amoA* copies g^{-1} soil), and then numbers decreased to 1.2×10^8 *amoA* copies g^{-1} soil at T3.

For the *amoA* AOB gene, significant increase was shown from T0 (2.2×10^7 *amoA* copies g^{-1} soil) to T2 (freeze-thaw, 2.5×10^8 *amoA* copies g^{-1} soil; control, 5.1×10^8 *amoA* copies g^{-1} soil). Numbers then decreased until 2.0×10^8 *amoA* copies g^{-1} soil in the freeze-thaw treated samples and 3.8×10^8 *amoA* copies g^{-1} soil in the non-frozen control samples at T3.

3.3.2.2 Nitrite and N_2O reducers

Regarding the genes involved in nitrite and N_2O reducers, the *nirK* gene copy numbers (4.7×10^8 - 6.2×10^9 copies g^{-1} soil) dominated over the *nirS* gene (4.7×10^6 - 1.2×10^7 copies g^{-1} soil) in both the Cu^{2+} contaminated and non Cu^{2+} contaminated samples during the incubation period as shown in Figure 15. Significant effects of Cu^{2+} contamination was detected on both *nirK* and *nirS* genes (*nirK*, 4.7×10^8 - 4.0×10^9 copies g^{-1} soil; *nirS*, 4.7 - 8.2×10^6 copies g^{-1} soil) when compared with the non Cu^{2+} contaminated soil samples (*nirK*, 6.1×10^8 - 6.2×10^9 copies g^{-1} soil; *nirS*,

5.3×10^6 - 1.2×10^7 copies g^{-1} soil, Figure 15 A, B). The phenomenon didn't change even under the freeze-thaw stress. However, there was no significant effect of Cu^{2+} contamination on *nosZ* gene copies according to Table 14.

In Cu^{2+} contaminated soil samples, *nirK* gene copy numbers in soils subsjected to freeze-thaw cycles (5.7 - 7.5×10^8 copies g^{-1} soil) were significantely lower than in non-frozen control samples (1.2 - 4.0×10^9 copies g^{-1} soil). Similar gene copy numbers were detected between freeze-thaw treated and control soil samples on *nirS* gene at T2 (around 5.8×10^6 copies g^{-1} soil).

Furthermore, significant differences at sampling time points were detected on *nirK* and *nosZ* gene copy numbers (see Table 14). In detail, a significant increase of *nirK* gene copy numbers was shown from T0 (4.7×10^8 copies g^{-1} soil) to T3 (freeze-thaw, 7.5×10^8 copies g^{-1} soil; control, 4.0×10^9 copies g^{-1} soil). For the *nosZ* gene, a significant increase was shown from T0 (1.1×10^7 copies g^{-1} soil) to T3 (4.9×10^7 copies g^{-1} soil) in non-frozen control samples. Conversely, a significant increase was detected from T0 to T2 (4.2×10^7 copies g^{-1} soil) and then numbers decreased to 3.3×10^7 copies g^{-1} soil at T3 in samples which had been subjected to freezing and thawing.

In non Cu^{2+} contaminated soil samples, freeze-thaw cycles resulted in significant differences in *nirK* and *nosZ* gene copy numbers (see Table 14). Briefly, significantly lower gene copy numbers were detected in the freeze-thaw treated soil samples (*nirK*, 0.7 - 2.4×10^9 copies g^{-1} soil; *nosZ*, 1.8 - 2.6×10^7 copies g^{-1} soil) than in the non-frozen control samples (*nirK*, 1.5 - 6.2×10^9 copies g^{-1} soil; *nosZ*, 4.2 - 5.3×10^7 copies g^{-1} soil). Similar *nirS* gene copy numbers were detected between freeze-thaw treated and control soil samples at T2 around 6.5×10^6 copies g^{-1} soil. Nevertheless, significant differences of sampling time points and the interaction between free-thaw stress and sampling times were detected on both *nirK* and *nosZ* gene copy numbers (see Table 14).

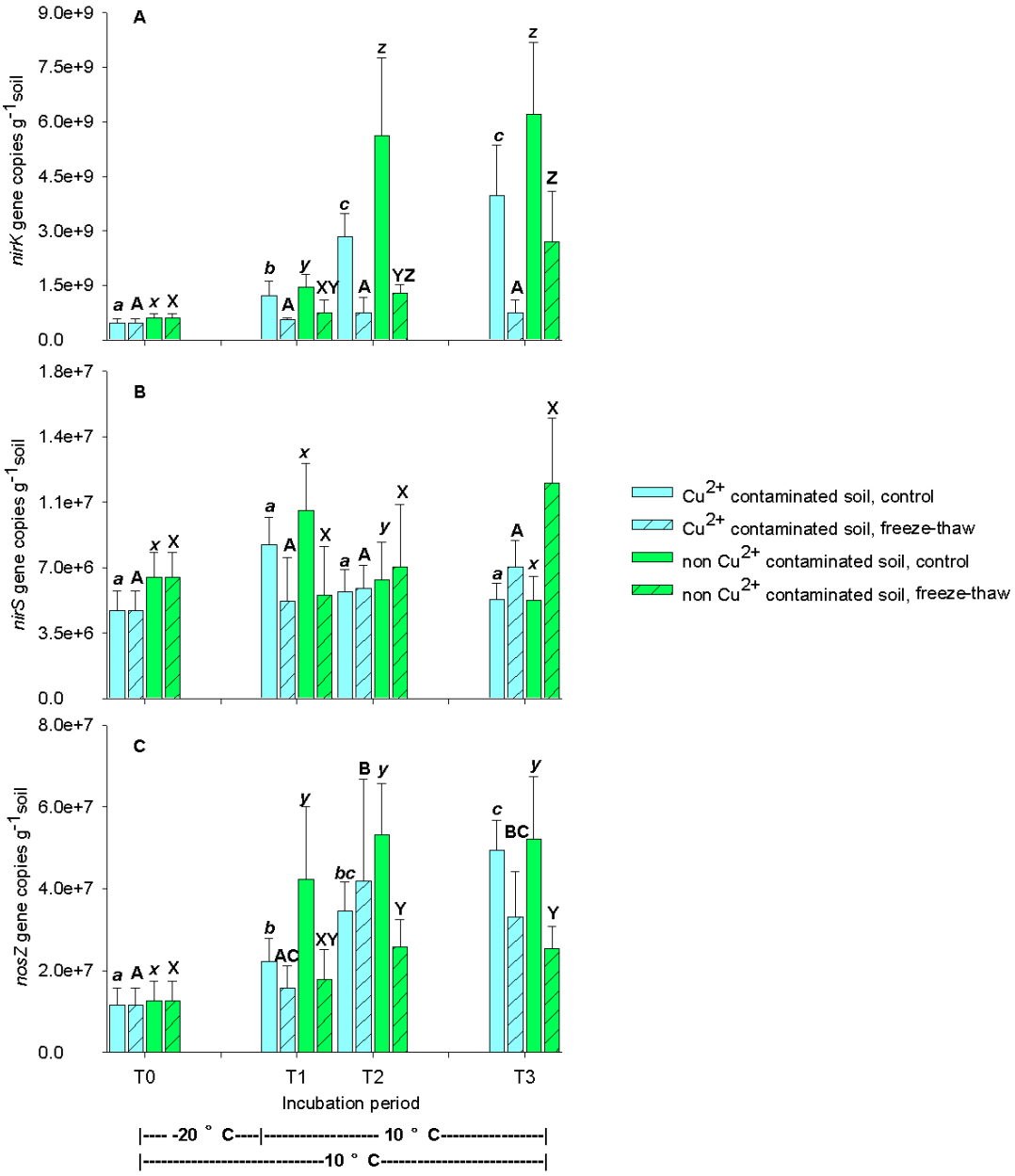

Figure 15 Abundance of nitrite and N_2O reducers related genes *nirK* (A), *nirS* (B) and *nosZ* (C) in E3. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). The error bars indicate that the standard deviation of the mean (n=5). Different letters display significant differences at the different time points.

In brief, *nirK* and *nosZ* gene copy numbers showed significant increases from T0 (*nirK*, 6.1×10^8 copies g^{-1} soil; *nosZ*, 1.3×10^7 copies g^{-1} soil) to T3 in both freeze-thaw treated (*nirK*, 2.7×10^9 copies g^{-1} soil; *nosZ*, 2.5×10^7 copies g^{-1} soil) and control samples (*nirK*, 6.2×10^9 copies g^{-1} soil; *nosZ*, 5.2×10^7 copies g^{-1} soil). Similar *nosZ* gene abundances were detected between T2 and T3 in both freeze-thaw treated (5.2 - 5.3×10^7 copies g^{-1} soil) and control samples (2.5 -2.6×10^7 copies g^{-1} soil).

Table 14 Statistical evaluations of nitrite and N_2O reducers related genes using ANOVA in E3. The *p* values describe the impacts and interactions of sampling time point, soil treatments and Cu^{2+} contamination on nitrite and N_2O reducers related functional genes (*nirK*, *nirS* and *nosZ*). Significant impacts or interaction are marked by asterisks (*) ($p<0.05$).

Factor	*p* values		
	nirK	*nirS*	*nosZ*
Total			
Time (T0, T1, T2,T3)	0.00 *	0.20	0.00 *
Treatment (freeze-thaw, control)	0.00 *	0.79	0.00 *
Cu^{2+} (contaminated, non contaminated)	0.00 *	0.01 *	0.29
Time × treatment	0.00 *	0.00 *	0.04 *
Time × Cu^{2+}	0.01 *	0.69	0.25
Treatment × Cu^{2+}	0.25	0.53	0.02 *
Time × treatment × Cu^{2+}	0.19	0.50	0.35
Cu^{2+} contamination			
Time (T0, T1, T2,T3)	0.00 *	0.19	0.00 *
Treatment (freeze-thaw, control)	0.00 *	0.49	0.13
Time × treatment	0.00 *	0.03 *	0.33
Non Cu^{2+} contamination			
Time (T0, T1, T2,T3)	0.00 *	0.64	0.00 *
Treatment (freeze-thaw, control)	0.00 *	0.81	0.00 *
Time × treatment	0.00 *	0.00 *	0.04 *

3.3.3 Community structure of ammonia oxidizers and selected denitrifiers

3.3.3.1 Archaeal ammonia oxidizers

In all samples, the dominant T-RFs were TRF-65, TRF-230, TRF-374 and TRF-502. Significant influences of Cu^{2+} contamination were detected in the low abundant T-RFs (Cu^{2+} contamination, TRF-65, 14 - 16%, TRF-502, 12 - 13%; non Cu^{2+} contamination, TRF-65, 13 - 14%, TRF-502, 13 - 15%) as shown in Figure 16.

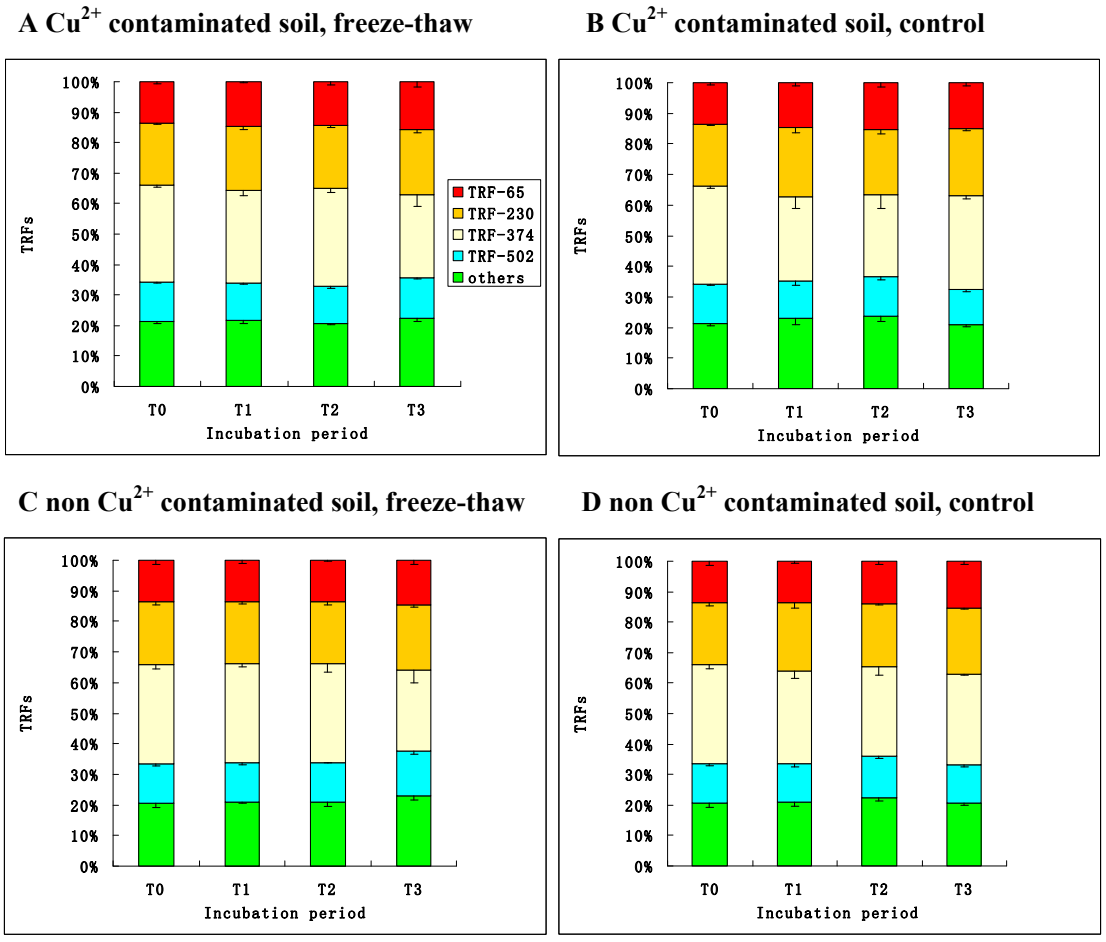

Figure 16 Dominant T-RFs contribute to the *amoA* AOA gene diversity. T-RFs contributing to below 5% are summarized as others. The relative abundance of the dominant peaks is expressed as percentage of the total amount of T-RFs. Samples were taken at T0 (the day before freezing), T1 (first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). Error bars indicate standard deviation of mean (n = 5).

The structure of the *amoA* AOA community shifted at different time points under different treatments (see Figure 16). Briefly, in freeze-thaw treated soil samples, a decrease of TRF-374 was shown from T0 (32%) to T3 (Cu^{2+}, 26%; non Cu^{2+}, 27%). However, decrease of TRF-374 was detected from T0 (32%) to T2 (Cu^{2+}, 27%; non Cu^{2+}, 29%), and then it increased until 30% at T3 in the non-frozen control samples. Moreover, an increase of TRF-230 was shown from T0 (20%) to T3 (22%) in each treatment.

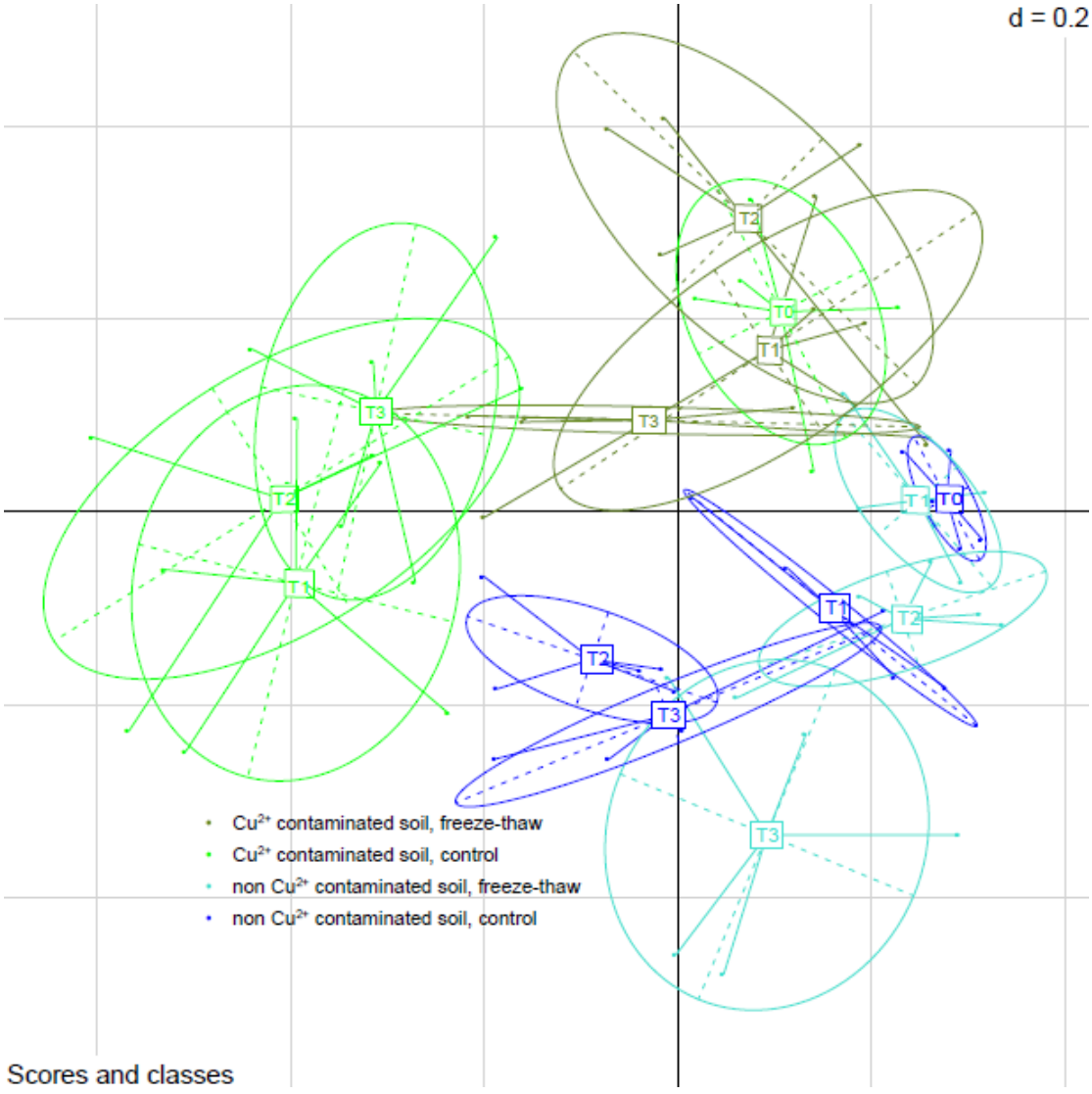

Figure 17 BGA of *amoA* AOA gene distribution. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing).Declared variances for component 1 on the horizontal axis and component 2 on the vertical axis are 39.5% and 22.2%, respectively.

Figure 17 shows that 61.7 % of the *amoA* AOA gene distribution variance could be explained using component 1 and component 2. Significant effects of Cu^{2+} contamination were detected shown by component 1, while, weak freeze-thaw influences were shown on component 2.

In the Cu^{2+} contaminated soil samples, a significant freeze-thaw influence was detected at T1 and T2. However, there was no difference shown in the freeze-thaw treated samples during the incubation period. Significant separation of *amoA* AOA gene distribution was only found between T0 and other sampling days (T1, T2 and T3) in the non-frozen control samples.

In non Cu^{2+} contaminated soil samples, a significant freeze-thaw influence was detected at T1 and T2. Furthermore, clear separation in freeze-thaw treated samples was shown between T1 and T3; whereas a similar separation was detected between T1 and T2 in non-frozen control samples. There was also a clear community shift found between T0 and other sampling time (T1, T2 and T3) in the non-frozen control samples. However, the shift was only detected between T0 and T3 in the samples which had been subjected to freezing and thawing.

3.3.3.2 N_2O reducers

4 dominant T-RFs, TRF-140, TRF-222, TRF-236 and TRF-265, were detected on *nosZ* gene as shown in Figure 18. Significant influences of Cu^{2+} contamination were shown on TRF-236 and TRF-265 (Cu^{2+} contamination, TRF-236, 4 - 10%, TRF-265, 28 - 39%; non Cu^{2+} contamination, TRF-236, 5 - 11%, TRF-265, 25 - 33%, Figure 18).

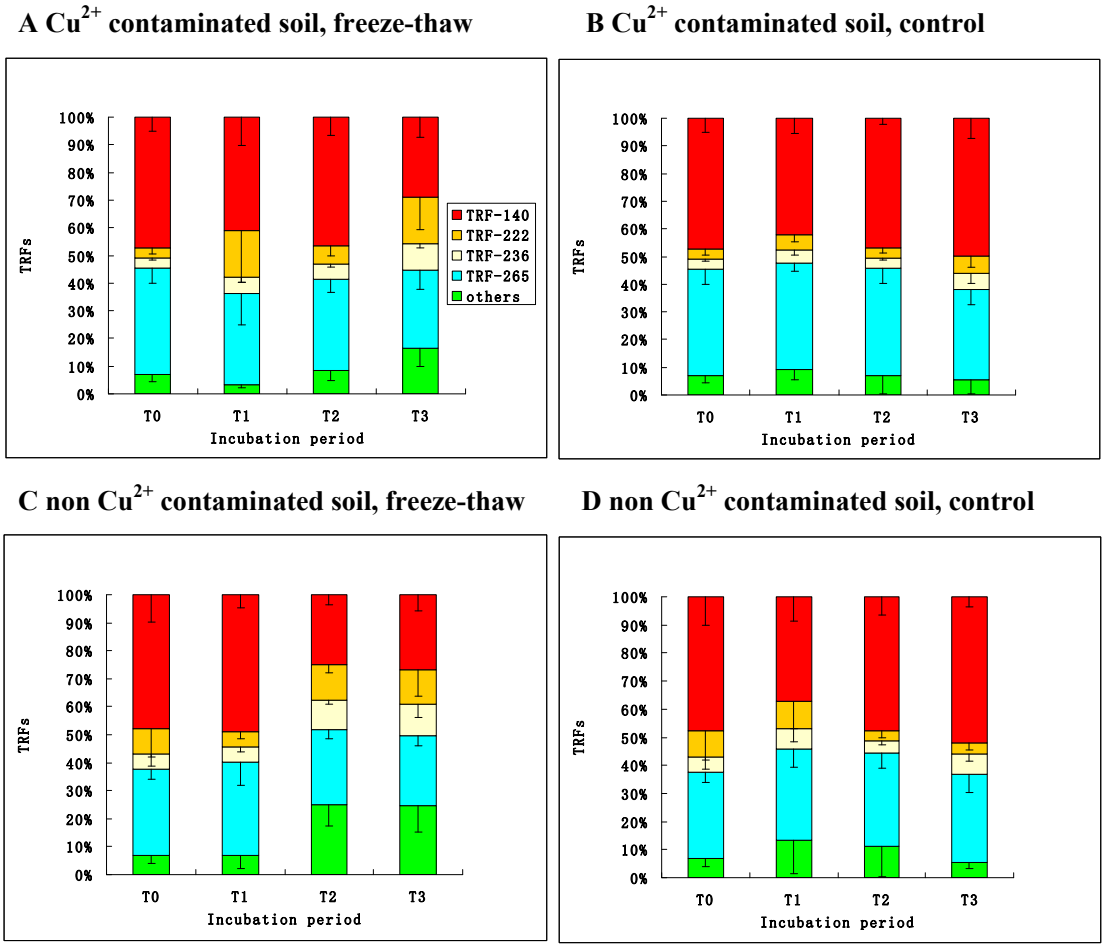

Figure 18 Dominant T-RFs contribute to the *nosZ* gene diversity. T-RFs contributing to below 5% are summarized as others. The relative abundance of the dominant peaks is expressed as percentage of the total amount of T-RFs. Samples were taken at T0 (the day before freezing), T1 (first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). Error bars indicate standard deviation of mean (n = 5).

In Cu^{2+} contaminated soil samples, significant influences of freeze-thaw stress were shown on TRF-140, TRF-222 and TRF-236 (see Figure 18). In detail, a higher proportion of TRF-140 was found in the non-frozen control samples (42 - 50%) than in soils which had been subjected to freezing and thawing (28 - 47%). On the contrary, higher proportions of TRF-222 and TRF-236 were detected in the freeze-thaw treated samples (TRF-222, 4 - 17%; TRF-236, 4 - 10%) than in the non-frozen control samples (TRF-222, 4 - 6%; TRF-236, 4 - 6%).

Furthermore, decreased proportion of TRF-140 were detected in samples which had been subjected to freezing and thawing from 47% at T0 to 28% at T3, while no difference was found in the non-frozen control samples. There was also no difference in the proportion of TRF-236 shown in the non-frozen control samples. However, an increased proportion of TRF-236 was detected from 4% at T0 to 10% at T3 in samples which had been subjected to freezing and thawing. A significant decrease in the proportion of TRF-265 was detected from 38% at T0 to T3 (freeze-thaw, 28%; control, 32%).

In non Cu^{2+} contaminated soil samples, significant influences of freeze-thaw cycles were found on both TRF-140 and TRF-236. Higher proportions of TRF-140 were detected in the non-frozen control samples (37 - 49%) than in the freeze-thaw treated samples (25 - 49%). On the other hand, a higher proportion of TRF-236 was detected in samples which had been subjected to freezing and thawing (5 - 11%) than in the non-frozen control samples (4 - 7%). Furthermore, a decreased proportion of TRF-140 was detected in freeze-thaw treated samples from 48% at T0 to 27% at T3. A decreased proportion of TRF-140 was shown in the non-frozen control samples from 48% at T0 to 37% at T1 and then it increased again to 53% at T3. The proportion of TRF-222 was elevated in samples which had been subjected to freezing and thawing from 9% at T0 to 12% at T3 but it decreased from 9% at T0 to 4% at T3 in the non-frozen control samples. For TRF-236, increased proportions were detected from 5% at T0 to T3 (freeze-thaw, 11%; control, 7%). For TRF-265, however, decreased proportions were only detected in samples which had been subjected to freezing and thawing from 31% at T0 to 25% at T3.

In Figure 19, it shows that 74.1% of the *nosZ* gene distribution variance could be explained using component 1 and component 2. According to component 1, a significant effect of freezing and thawing was detected at T1 and T3 in the Cu^{2+} contaminated soil samples and at T2 and T3 in the non Cu^{2+} contaminated soil samples. In the Cu^{2+} contaminated soil samples, significant differences of the *nosZ*

gene distribution were found between T3 and other sampling days (T0, T1 and T2) in samples which had been subjected to freezing and thawing. Similar differences were also found between T0 and T1 in the non-frozen control samples. Moreover, in the non Cu^{2+} contaminated soils, clear separation was shown between samples which had been subjected to freezing and thawing from T0 and T1 to T2 and T3. There was no difference among the non-frozen control samples.

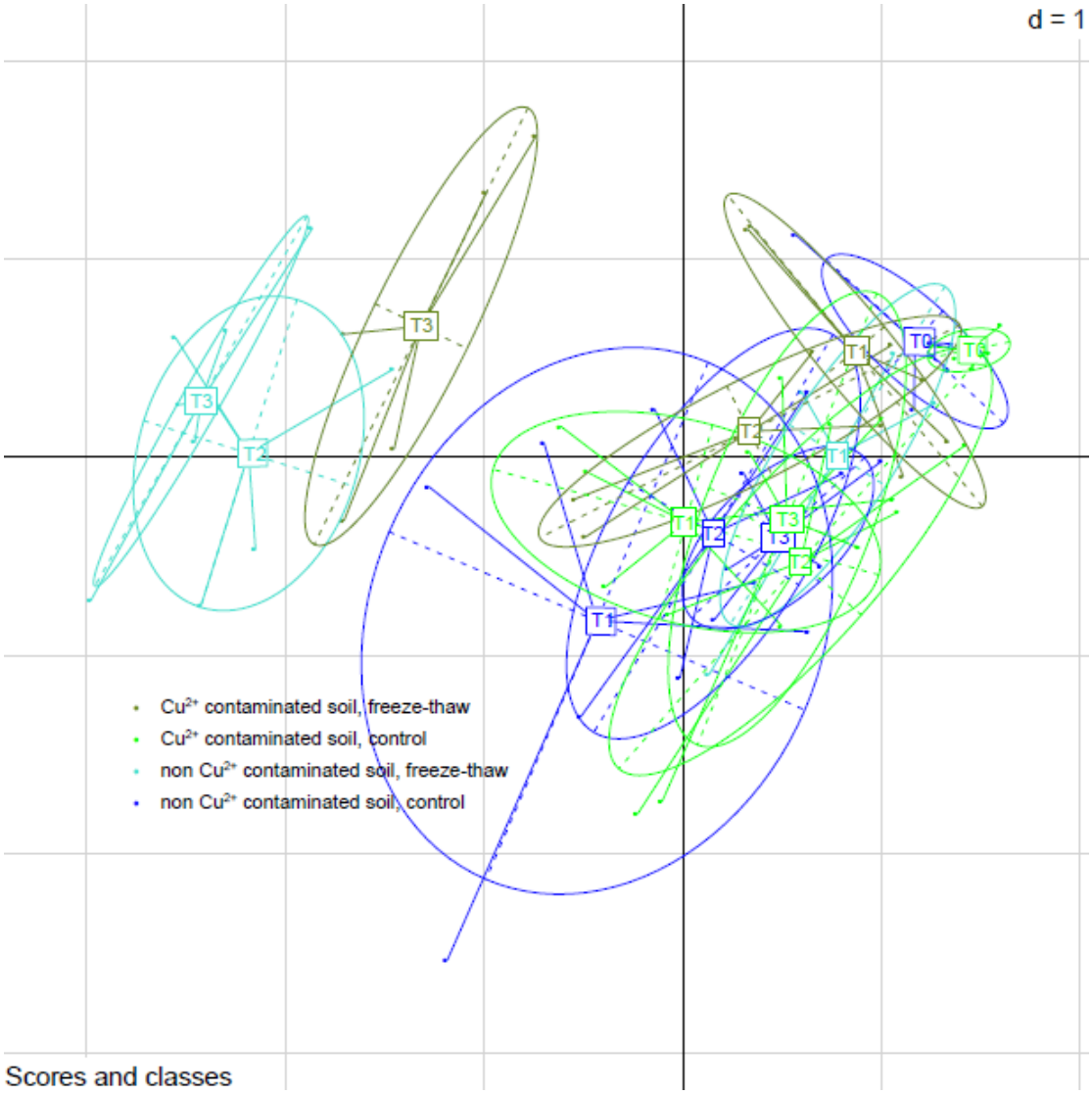

Figure 19 BGA of *nosZ* gene distribution. Samples were taken at T0 (the day before freezing), T1 (the first day after thawing), T2 (the third day after thawing) and T3 (the seventh day after thawing). Declared variance for component 1 on the horizontal axis and component 2 on the vertical axis are 63.9% and 10.2%, respectively.

4 Discussion

This thesis investigated the influence of freezing and thawing on nitrification and denitrification. Soil with different plant residues was used in order to compare how amendments with different C and N inputs influence freeze-thaw effects. Soil with Cu^{2+} contamination was studied to demonstrate how freeze-thaw stress influenced the impact of Cu^{2+}.

4.1 Influences of amendments on nitrification and denitrification

In this research, the amendments of legume-grass mixtures and wheat straw were shown to cause very different reactions in terms of soil chemical content and soil microbial gene abundances, especially under freeze-thaw stress.

Due to the lower C/N ratio in the legume-grass mixtures than in wheat straw, higher NH_4^+ and NO_3^- contents were detected in the legume-grass mixtures than in the soils amended with wheat straw in both E1 and E2. That might be because the legume-grass mixtures could be easily used by soil microorganisms as the inorganic N provided more available N for microbial activities (Publication I). NO_3^- is one of the products of nitrification. It is also a thermodynamically-favourable N electron acceptor for denitrification when O_2 is limited (Clark *et al.* 2009, Heylen *et al.* 2006). The results indicated that soils with legume-grass mixture amendments influenced both nitrification and denitrification abundance. Such a difference was not detected in wheat straw amended soil samples. That might have been because with a higher C/N ratio, wheat straw showed extremely low NO_3^- concentration in E1 (3.2 - 5.5 μg g^{-1} soil) and E2 (0.06 - 0.14 μg g^{-1} soil). Chandra and Bollen (1960) supported the results of this study that wheat straw additions prevented the decreases in total N.

In E1, higher archaeal *amoA* (AOA) than bacterial *amoA* (AOB) gene copies were found in wheat straw amended soil samples. On the other hand, higher *amoA* AOB than *amoA* AOA gene copy numbers were detected in legume-grass mixtures amended samples which added the same C but higher N content. However, in E2, higher *amoA* AOA gene abundances were detected in both amendments-treated soil with the same N but different C content. Therefore, it was concluded that the trend of increasing archaea *amoA* gene numbers (AOA) but decreasing bacterial *amoA* gene (AOB) abundances in the legume-grass mixtures amended samples coincided with the decreased amount of amendment. In E1 it was 2g and in E2 it was 1/3g. These factors decreased the C and N content in the soil. Additionally, the increasing *amoA* AOA gene abundances and decreasing *amoA* AOB gene abundances in soils with wheat straw amended samples concurred with an increased amount of amendment. In E1 it was 2g and in E2 it was 4g. These factors increased the C and N content in the soil. Zavalloni *et al.*(2011) supported these results. They also found that wheat straw can significantly increase microbial C and N, but will decrease the soluble organic N. Additionally, a fast recovery of the *nirK* gene copy numbers was detected in the legume-grass mixtures amended samples which was also found by Sharma *et al.* (2006). The wheat straw amended soil also showed a decrease of the *nirK* gene copies in the control samples. Interestingly, higher *nirK* and *nosZ* gene copy numbers were detected in soil samples amended with legume-grass mixture than in the wheat straw treated samples in E1, while the total opposite was found in E2. This indicated that C availability was correlated with an increasing abundance of denitrifiers (Publication I).

4.2 Influences of freezing and thawing on nitrification and denitrification

It has been generally accepted that freezing and thawing cycles release organic C and N from soil and increase the microbial nitrogen transformation processes (Matzner and Borken 2008). Denitrification rates and the proportions of N_2O and other greenhouse gases produced by denitrifying microbes can vary dependent upon

numerous environmental factors, such as pH, carbon content, NO_3^- and NH_4^+ availability, soil moisture, pore structure, aeration, temperature, freezing-thawing, and drying-wetting events (Philippot *et al.* 2007).

Based on this research, generally higher gene copy numbers of *nirK* compared to the *nirS* gene have dominated in nitrite reduction. Those dominations were independent of amendments and treatments as shown in E1 and E2. The higher *nirK* gene copy numbers could reflect a possible niche differentiation for microbes harboring one of the functionally redundant nitrite reductases, where *nirK*-possessing bacteria could preferentially colonize habitats with more available C and N (Publication I). This trend has also been observed comparing hotspots in soils exhibiting higher amounts of available C and N with low activity areas (e.g., rhizosphere vs. bulk soil) (Sharma *et al.* 2005). Surprisingly, no visible significant influence of freeze-thaw stress was detected on both *nirK* and *nirS* genes in E1 and E2. This might have been because the availability of C and N in the samples was not subjected to freezing and thawing stresseson nitrite reductase. For the *nosZ* gene, significant lower gene abundances were detected only in the frozen samples treated with wheat which had a higher C content as in E2. That might have been because higher C contents accelerated the freeze-thaw effect by reducing the *nosZ* gene abundance.

In this thesis, the freeze-thaw event brought more influences to bear upon AOB than upon AOA abundances. This resonates well with the findings of Schleper *et al.* (2005) and Valentine (2007) who presumed that archaea are more tolerant to stress conditions than bacteria. Fierer *et al.* (2009) pointed out that there was an inseparable correlation between temperature and AOB community composition. Soils exposed to similar mean annual temperatures tended to have similar AOB communities. Several conditions such as a low pH value, heavy metal contamination and other stresses play an important role in an increasing the tolerance of archaea (Attard *et al.* 2010, Gubry-Rangin *et al.* 2010, Mertens *et al.* 2009). The versatile metabolisms of AOA and AOB provided an eventual mixotrophic growth (Prosser and Nicol 2008, Schleper

2010). The influences of freezing and thawing resulted in an increase in AOA/AOB ratios in both legume-grass mixtures and wheat straw treated soil samples in E1. Leininger *et al.* (2006) showed that AOA/AOB ratios increased when there was decreasing availability of C and N. AOA might be more closely attached to the soil particles which might result in a certain protection for AOA compared to AOB. In the case of ammonia oxidizers, it was mainly AOB that benefitted from the increased availability of NH_4^+ in soil (Schauss *et al.* 2009). However, there was no relationship detected between AOB and NH_4^+ in E2. This might be because the available O_2 prevented rapid NH_4^+ oxidation.

The availability of C content in soils not only plays an important role in the global C cycle but also determines denitrification activities in the N cycle (Mrkonjic Fuka *et al.* 2009, Phillips 2008). In this thesis, the quality of C between the two amendments was different. The wheat straw had a higher C/N ratio and contained a lot of cellulose. It is notable that the legume-grass mixture in which was detected a lower C/N ratio, can be easily degraded by microorganisms. This research indicated that higher WEOC content was related to higher N content in the soil samples in E1. The N content also influenced the availability of WEOC. Significant differences were found on legume-grass mixture amended samples between freeze-thaw treated and non-frozen control samples in terms of WEOC in E1 rather than in E2 which was amended with less N.

Publication I concluded that WEOC affects N turnover in two ways: on the one hand it stimulates protease activity and the formation of inorganic N, and on the other hand it is also a prerequisite for denitrification. There was no difference between freeze-thaw treated and non-frozen control samples in terms of N-related soil chemicals in E2. That might have been because under the lower N conditions the difference of available NH_4^+ and NO_3^- concentrations on freeze-thaw stress was not large enough to be detected. Sehy *et al.* (2004) also found higher microbial gene abundances in samples with wheat straw additions. This might have been because of

the higher amounts of WEOC which resulted in anaerobic microbe activity during the thawing period. High amounts of NH_4^+ during mid-winter thawing periods are related to the higher availability of C which increased soil protease activity (Mrkonjic Fuka *et al.* 2009). Therefore, mineralized inorganic N might be used by microbes for biomass generation and was no longer available as NH_4^+ or NO_3^-.

It is generally accepted that the amount of available inorganic N is one of the factors regulating denitrification rates in soil (Phillips 2008). This is not only true for the vegetation period but can also be extended to the winter period with freezing-thawing. In permafrost regions, soil thawing is often accompanied by an accelerated release of nutrients and the emission of greenhouse gases such as N_2O, NO, CO_2 and CH_4. The release is adjusted by the water saturation and the availability of degradable C and NO_3^- in those soils (Elberling *et al.* 2010, Repo *et al.* 2009).

This study shows that significantly higher NH_4^+ content was detected in freeze-thaw treated soils with legume-grass mixture amendments in E1 and E2. It indicated an enhanced mineralization of organic N and an increased release of NH_4^+ through frost weathering and cell disruption. Increasing N_2O emissions from soils are associated with a growing N inputs by fertilization of agricultural soils (Skiba and Smith 2000). Both laboratory and field studies have observed an increased contribution for nitrification on the total N_2O production in correlation with increasing NH_4^+ concentrations and nitrified activity after fertilization (Müller *et al.* 1998, Schuster and Conrad. 1992).

The thesis also found that in the freeze-thaw treated soil samples, lower NO_3^- concentrations were detected in those soil samples amended with legume-grass mixtures in E1 and E2. this is similar to the results by Clark et al. (2009) suggested that nitrification was the source of NO_3^- accumulation in frozen soils, and this process was consequently limited by NH_4^+ availability. Therefore, higher NH_4^+ concentrations are often associated with decreased NO_3^- concentrations (Elliott and Henry 2009).

According to E1 and E2, the amount and quality of applied N showed distinct effects on the N cycling related gene abundances during freezing and thawing events. This was supported by previous studies which showed a close relationship between freeze-thaw stresses and the different transformational processes of the N cycle (Nannipieri and Eldor 2009). However, this project only focused on the DNA level, and the effects of freezing and thawing on the activity of nitrification and denitrification were unknown. The research was based on the mRNA level reported by Bollmann *et al.* (2005) who suggested that the fast recovery of ammonia oxidizing activity was detected after addition of NH_4^+. Therefore, a higher activity of ammonia oxidizers was correlated with a higher NH_4^+ concentration in soil. This increases the nitrite oxidizers since ammonia oxidizers provide the substrate for the second process of nitrification, where NO_3^- is formed. The increasing nitrite oxidizers are, in turn, important for denitrification because NO_3^- is the thermodynamically-favourable N electron acceptor in denitrification processes (Heylen *et al.* 2006).

4.3 Influences of Cu^{2+} on nitrification and denitrification

The legume-grass mixtures were efficient N suppliers. They were also used in the E3 experiment with Cu^{2+} contamination. It can be quickly mineralized and supplied the released compounds for nitrification and denitrification during winter times as a result of freezing and thawing as shown in Publication I. Due to binding the organic matter fraction in soils, Cu^{2+} availability was significantly influenced by freezing and thawing. That might have been because Cu^{2+} becomes more bio-available after freeze-thaw events. However, by controlling the breakdown of organic matter, high Cu^{2+} content can cause harmful effects on soil microorganisms, net fluxes and the amounts of soil C and N. This happens through decomposition, mineralization and immobilization processes (Nannipieri *et al.* 2003).

The results of this study supported the hypothesis that Cu^{2+} degraded over time, but freezing and thawing reduced the speed of Cu^{2+} contamination on T1. The opposite

results were shown for WEOC and NH_4^+, NO_3^- contents of Cu^{2+} contaminations in E3. That might have been because the influence of Cu^{2+} contamination was more dominated than freezing and thawing.

Cu^{2+} contamination had strong effects on the gene abundances involved in N cycling and the corresponding N transformations during freezing and thawing events. Nitrification process can be affected by many factors including pH, moisture, temperature and organic matters (Breuer *et al.* 2002, Dancer *et al.* 1973, Innerebner *et al.* 2006). This research found that ammonia-oxidizing bacterial (AOB) not ammonia-oxidizing archaeal (AOA) had more abundance of *amoA* gene in both Cu^{2+} contaminated and non Cu^{2+} contaminated soil. The phenomenon was supported by Mertens *et al.* (2009) that AOB, but not AOA, mediate recovery of nitrification after zinc (Zn) contamination. However, the pristine soils showed approximately equal *amoA* (AOA and AOB) gene copy numbers and transcript levels. Höfferle *et al.* (2010) also pointed out that quantification of *amoA* genes demonstrated greater abundances of bacterial than archaeal *amoA* genes throughout the soil profile at the polluted site, while bacterial *amoA* genes at the unpolluted site were below the detection limit.

The T-RFLP analysis of *amoA* (AOA) gene detected less community shift in the freeze-thaw treated samples than in the control. Cu^{2+} contamination related community shifts of *amoA* AOA gene were detected in non-frozen soil samples but not in freeze-thaw treated samples. That might have been because the Cu^{2+} availability was strongly influenced by freeze-thaw stress.

Higher Cu^{2+} concentrations led to lower *amoA* AOA than *amoA* AOB gene transcription and expression in frozen soil samples. This is supported by Leininger et al. (2006) who suggested that AOA might be more closely attached to soil particles; this might result in a certain protection for AOA compared with AOB. Moreover, the presence of high Cu^{2+} concentrations had a potentially negative effect on several aspects of microbial presence such as diversity, abundance, activity and composition

(Bååth 1998, Brookes 1995, Giller *et al.* 1998). In this research, although the freeze-thaw stresses strongly reduced the influence of Cu^{2+} contamination, they didn't significantly change the AOA microbe community. The high sensitivity of microbial processes in contaminated soil was influenced by environmental soil parameters (Sauvé *et al.* 1999). For example, the response of the microbial community to additional stressors such as pesticide addition, freeze-thaw or dry-wet cycles depends on the history of the soil samples and the type of stressor applied (Tobor-Kapłon *et al.* 2006b). Mertens *et al.* (2007) supported these results. They found that neither resistance nor resilience to these stressors was caused by adaptation of the nitrifying communities to elevated Zn concentrations in long-term contaminated soils.

The results of this investigation also showed that freeze-thaw and Cu^{2+} contamination stresses interacted with each other on *amoA* AOA gene copies during the whole incubation period. Interestingly, no significant effect of Cu^{2+} contamination was detected on both archaeal and bacterial *amoA* gene copy numbers in E3. Freeze-thaw cycles showed significant lower bacterial *amoA* gene (AOB) abundances in Cu^{2+} contaminated soil samples compared to non Cu^{2+} contaminated samples. That might have been because the influence of freezing and thawing was promoted by Cu^{2+} contamination. Additionally, no significant differences in *amoA* AOA gene copy numbers were detected between freeze-thaw treated and non-frozen control samples. In other words, AOB were more affected by the freeze-thaw events compared with AOA.

Several bacteria genera showed high tolerance for heavy metals (Schleper *et al.* 2005, Valentine 2007). Bååth (1998) and Mergeay *et al.* (2003) noticed that bacterial communities can increase their tolerance of heavy metals. This research found that significant higher *nirK* and *nirS* gene copy numbers were detected in non Cu^{2+} contaminated than in Cu^{2+} contaminated soils. There are several possible reasons for this tolerance, such as the substitution of sensitive strains by tolerant ones, the spread of resistant genes, and genetic modifications for producing heavy metal resistance

(Smalla and A.Sobecky 2002). Johansson *et al.* (1998) and Throbäck *et al.* (2007) supported these results. They also suggested that the number of NirK-type denitrifiers was negatively correlated with increasing concentrations of Ag^+ in soils.

Moreover, *nirK* gene copy numbers were strongly influenced by both the Cu^{2+} contamination and freeze-thaw effects. Where higher *nirK* and *nirS* gene abundances were detected lower WEOC and NO_3^- concentrations were shown. This confirms the hypothesis that the lower amounts of C and NO_3^- in the soil samples can be explained by higher denitrification activities. Sehy et al. (2004) also suggested that the availability of C substrates play an important role in freeze-thaw related N emissions.

However, freeze-thaw influences on *nirS* gene were detected at T3 and Cu^{2+} contamination effects were rare during the whole thawing period. When comparing the effects between freeze-thaw and Cu^{2+} contamination stresses on *nosZ* gene copy numbers, the principal influence of Cu^{2+} contamination was detected only in the non-frozen treatment. The reason for this might have been because the freeze-thaw stress strongly reduced the Cu^{2+} contamination influence. Significant freezing and thawing effects on *nosZ* gene copies was shown at T1 and T3 in Cu^{2+} contaminated soil samples and at T2 and T3 in non Cu^{2+} contaminated soil samples. That might be because Cu^{2+} contamination exacerbated the freeze-thaw effects.

4.4 Influences of sampling times on nitrification and denitrification

In this investigation, soils were sampled at the same site. This was cropped with potatoes and winter wheat in a rational crop rotation during the sampling period from September 2008 to May 2010. Due to the fast degradation of legume-grass mixtures found in the studies, pre-incubation time were shorted from two weeks in E1 and E2 to three days in E3. Therefore, sampling times in E1 and E2 were eleven days later than in E3, which led more C and N content to remain in the soils. The non Cu^{2+} contaminated soil in E3 were compared with the legume-grass mixtures amended soil

in E1, in order to detect how the shorter pre-incubation period influenced nitrification and denitrification.

Although differences between WEOC content in E1 and E3 at T0, the results indicated that similar tendencies for change were detected during the experiment. However, NH_4^+ and NO_3^- contents were different, even opposite between E1 and E3. Clark *et al.* (2009) and Elliott (2009) supported these results. They found that NO_3^- accumulation in frozen soils was limited by NH_4^+ availability and that higher NH_4^+ concentrations were often associated with increased NO_3^- concentrations.

More nitrification and denitrification related gene abundances were detected at T0 in E3 than in E1, except the *nosZ* gene. A shorter pre-incubation time showed no influence on the increasing numbers of nitrification related gene abundances. The AOA/AOB ratios were lower than 1 during the incubation period. Leininger *et al.* (2006) supported these results as they found that AOA/AOB ratios increased with decreasing availability of C and N. In other words, lower AOB gene abundances were related with higher C and N.

Higher *nirK* gene abundances were detected in E3 than in E1, which is supported by the findings of Sharma *et al.* (2006) who suggested that fast recovery of *nirK* gene copy numbers were related to high amounts of available C and N. The short incubation period had no clear influence on *nirS* and *nosZ* genes. However, in E3 the *nosZ* gene was detected at lower gene abundances under the freeze-thaw stress condition. On the other hand, higher gene copy numbers were found in E1 under the same situation. That might have been because of the higher C and N content; the freeze-thaw stress enhanced the denitrification related N_2O emission and reduced N_2O reduction (Groffman and Tiedje 1991).

4.5 Conclusions and perspectives

In this study, the effects of freezing and thawing on nitrification and denitrification in an arable soil were investigated. In order to observe the interactive influences among the amendments and freeze-thaw stresses on soil nitrification and denitrification, different plant residues - a legume-grass mixture and wheat straws - were amended into the soils. These contained variable amounts of C and N. Moreover, reflecting a widely-used fungicide in agricultural practice, $CuCl_2$ was added into the test soil as heavy metal contaminant.

The results revealed that the N stored in legumes-grass mixtures can be mineralized, nitrified, and denitrified quickly during winter times. Due to different plant residues with different C to N ratios used in E1 and E2, a tight relationship between the C and N cycles was indicated. After exposure to freezing and thawing, the N transformation related gene abundances and performance of the microbes were clearly influenced by the organic C content. Different plant residues have a stronger impact than freezing and thawing. Therefore, the finding of E2 might help to improve agricultural management in cover crops and the application of green manure in autumn and winter.

In E3, the Cu^{2+} contamination did not only affect organic C and N content of the soil, but also the abundance of genes in N cycling. An effect of Cu^{2+} can be found in N transformation during the freezing and thawing as well. Cu^{2+} contamination accelerated the freeze-thaw effect and the freeze-thaw stress reduced the influence of Cu^{2+} contamination on soil during the whole incubation period.

Although this research has revealed significant influences of freezing and thawing on nitrification and denitrification under different amendments and heavy metal contamination, there is still a need to consider other environmental factors in future research, such as frequency of freeze-thaw cycles, different water conditions and the

oxygen content in soil. These aforementioned conditions may influence the activity of nitrifiers and denitrifiers, which directly contributes to the nitrification and denitrification of soils.

The molecular analyses in this investigation were focused at the DNA level. They were valuable and sensitive tools that effectively detected soil microbial communities. However, there is still some lack of knowledge about the transcription and translation in the processes of nitrification and denitrification at the RNA level.

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6 Appendixes

6.1 Figure legends

6.2 Table legends

6.3 List of abbreviations

amoA	gene encoding the α-subunit of the ammonia monooxygenase
amoB	gene encoding the β-subunit of the ammonia monooxygenase
amoC	gene encoding the γ-subunit of the ammonia monooxygenase
AMO	ammonia monooxygenase
AMMI	additive main effects and multiplicative interaction
ANOVA	analysis of variance
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
ATP	adenosine triphosphate
BGA	between group variance
BSA	bovine Serum Albumin
$CaCl_2$	calcium chloride
CH_4	methane
CO_2	carbon dioxide
cNOR	cytochrome c-dependent nitric oxide reductase
CT	threshold cycle
$CuCl_2$	copper chloride
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Eff	amplification efficiency
FAM	Carboxyfluorescein
Fe-	iron-based
HAO	6-hydroxylamine oxidoreductase
hao	gene encoding the hydroxylamine oxidoreductase
HSD	Honestly Significant Difference
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy

Mo-	molybdenum-based
MQH_2	menahydroquinone
N_2	nitrogen gas
NAP	periplasmic nitrate reductase
napA	gene encoding the subunit of periplasmic nitrate reductase
napB	gene encoding the subunit of periplasmic nitrate reductase
NAR	membrane-bound nitrate reductase
narG	gene encoding the subunit of membrane-bound nitrate reductase
narH	gene encoding the subunit of membrane-bound nitrate reductase
narI	gene encoding the subunit of membrane-bound nitrate reductase
N_2H_4	hydrazine
NH_2OH	hydroxylamine
NH_3	ammonia
NH_4^+	ammonium
NirK	copper-containing nitrite reductase
nirK	gene encoding the copper-containing nitrite reductase
NirS	cytochrome cd1 nitrite reductase
nirS	gene encoding the cytochrome cd1 nitrite reductase
NOR	nitric oxide reductase
NosZ	nitrous oxide reductase
nosZ	gene encoding the catalytic subunit of the nitrous oxide reductase
N_2O	nitrous oxide
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NXR	nitrite oxidoreductase
NxrA	α-subunit of nitrite oxidoreductase
nxrA	Gene encoding the α-subunit of nitrite oxidoreductase
NxrB	β-subunit of nitrite oxidoreductase

nxrB	Gene encoding the β-subunit of nitrite oxidoreductase
O_2	oxygen
PCR	polymerase chain reaction
qCu_ANOR	hydroquinol and cytochrome-dependent nitric oxide reductase
QH_2	ubihydroquinone
qNOR	hydroquinol-dependent nitric oxide reductase
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SG	SYBR Green
TNb	Total nitrogen
T-RFLP	terminal restriction fragment length polymorphism
T-REX	software for the processing and analysis of T-RFLP data
T-RFs	terminal restriction fragments
V-	vanadium-based
WEOC	water extractable organic carbon

7 Acknowledgments

First of all, I would like to give the deeply thanks to Prof. Dr. Michael Schloter who gave me this fantastic chance to work with my thesis and supported me with excellent guidance during my Ph.D. study. His patience and broad knowledge in science helped me to open my scientific view and finish this dissertation. I hope that I can follow the spirit of him on the science research.

Then, I want to take the special thanks to Dr. Kristina Kleineidam who give me excellent suggestions on the experiment designing and accomplishing and the paper writing. I learned two important skills which were really useful to do research from her: the patience and conscientious attitude.

Furthermore, I would give profuse thanks to Prof. Dr. Jean Charles Munch for the possibility to perform this Ph.D. thesis in his institute and his tender and amiable smile gives me deeply impression

In addition, I was really glad to thank all my colleagues in our group who give me warm help not only in my study but also in my daily life. Many thanks to Dr. Stefanie Schulz and Dr. Andrea Bannert who gave me great help during my thesis writing; to Dr. Xia Dong who always gave me some tips to avoid to walk in the dark; to Dr. Roland Fuss who suggested me to use my own thinking on the Ph. D. study; to Verena Hammerl who gave me warmly support during my thesis writing; to Rolf Schilling who always helped me to collect the soil sampling; to Gudrun Hufnagel for the scalar measurements and wonderful skill on pipette management; to Franz Buegger who always gave me quick result about the C and N content of plant; to Conny Galonska who gave me much help on DNA extractions.

I would like also to acknowledge the financial support from the China Scholarship Council (CSC) for my Ph.D. study and the chance they give to me to study in Germany. Most importantly, I want to express my deepest love and gratitude to my parents, especially my mother, thanks to their supports and encouragements during my lift led me always have confidence and courage to solve every difficulty.

8 Curriculum vitae

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