Winter expression of soil nitrogen cycle genes in agricultural soils representing the Boreal Atlantic Maritime climate

By © Victor Pablo Valdez

A Thesis submitted
to the School of Graduate Studies in partial fulfillment of the
requirements for the degree of

Master of Science in Environmental Science

Faculty of Science

Memorial University of Newfoundland

2019

St. John’s Newfoundland and Labrador

ABSTRACT

The expression of the soil nitrogen-fixing (*nifH*), ammonia oxidizing (archaea and bacteria *amoA*) and denitrifying (*narG*, *napA*, *nirK*, *nirS*, and *nosZ1*) genes are commonly used as indicators for these processes during the non-growing season. This study quantified the transcript abundance profiles of these genes by cDNA for Droplet Digital PCR in soils for the control (native vegetation) and four, regionally relevant crop production systems in Newfoundland. Soil parameters analysed were pH, total carbon, NH_4^+-N, NO_3^--N, and water-filled pore space. All genes quantified were expressed in winter suggesting that microorganisms were responding to minute changes in soil parameters; and that N-fixation and (de)-nitrification were co-occurring. Snowpack accumulation led to an increase in all transcript abundance profiles while pure alfalfa stands using mineral fertilizers had the lowest transcript abundance profiles. NO_3^--N and pH were negatively correlated to *nifH* gene expression, suggesting the latter is likely downregulated to balance growth in acidic soil conditions.

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ACKNOWLEDGEMENTS

I am grateful to Dr. Adrian Unc for being advised to conduct a Master of Science graduate thesis. I am also grateful for Dr. Linda Jewell for her invaluable support and knowledge throughout the research project timeline. Consideration is given to Agriculture and Agri-Food Canada's St. John's Research and Development Centre for the financial support and allowing me to use their experimental fields for downstream soil analysis and partake in important real-scenario work. I also thank Dr. Yuan Tao and Jamie Warren at Memorial University's Grenfell Campus Boreal Ecosystem Research Initiative laboratory for their help with the quantitation of the samples. Finally, I am thankful to the School of Graduate Studies for the financial support via the Graduate Fellowship program.

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LIST OF ABBREVIATIONS

Agriculture and Agri-Food Canada (AAFC)

A Horizon (Mineral Horizon)

Ammonia-oxidizing bacteria (AOB)

Ammonium-Nitrogen (NH_4^+-N)

Archaeal Ammonia-oxidizing (AOA)

Archaeal-amoA (AMOA)

Bacterial-amoA (AMOB)

B Horizon (Subsoil)

Biological Nitrogen Fixation (BNF)

Bulk Density (BD)

Canonical Correspondence Analysis (CCA)

Carbon (C)

Carbon Dioxide (CO_2)

DeNitrification-DeComposition (DNDC)

Dinitrogen (N_2)

Deoxyribonucleic Acid (DNA)

Droplet Digital PCR™ (ddPCR™)

Dry weight (DW)

Fall (F)

Freeze-Thaw Cycles (FTCs)

Gravimetric Moisture Content (GMC)

gram (g)

Greenhouse Gases (GHGs)

Hydrogen (H)

Linear Discriminant Analysis (LDA)

LFH Horizon (Litter, Folic and Hummus)

Methane (CH_4)

Microliter (µL)

Nanogram (ng)

Newfoundland and Labrador (NL)

Nitrate Leach and Economics Analysis on Stella (NLOS)

Nitrate-Nitrogen (NO_3-N)

Nitrite oxidizing bacteria (NOB)

Nitrite oxidoreductase (NXR)

Nitrogen (N)

Nitrogen-cycling (N-cycling)

Nitrous Oxide (N_2O)

O Horizon (Organic Horizon)

Oxygen (O_2)

Plough Layer (PL)

Polymerase Chain Reaction (PCR)

Reverse Transcription (RT)

Revolutions per minute (rpm)

Ribonucleic Acid (RNA)

Soil Water-Filled Pore Space (WFPS)

Spring (S)

Sub-layer (SL)

Total Carbon (TC)

Total Nitrogen (TN)

Total Porosity (St)

Treatments 0-4 (T 0-4)

Volumetric Moisture Content (VMC)

Winter (W)

1 GENERAL INTRODUCTION

The boreal forest, which extends principally through Alaska, Canada, Finland, Sweden, Russia, and Norway, is an ecozone covering approximately one third of the Earth's extant forests (Brandt, 2009). Boreal forest soils are major sinks for nitrous oxide (N_2O), carbon dioxide (CO_2), and methane (CH_4) (Adams et al., 1990; Bouwman, 1990; Gorham, 1991; IPCC, 2008). The release of these three main greenhouse gases (GHGs) is however prevented by the limited decomposition rates due to low temperatures (Deluca & Boisvenue, 2012; Toberman et al., 2010). In 2011, 3,435,600 ha of the Boreal Shield and Atlantic Maritime ecozones were under agricultural management (Statistics Canada, 2014), a portion of the ecozones' potential agricultural land (20,587,600 ha).

The Canadian agriculture market received an estimated 463,000 metric tonnes of N fertilizers shipments for the 2017-2018 (Statistics Canada, 2018). Fertilization of agricultural soils in the northern regions which experience seasonal freeze-thaw cycles (FTCs) lead to excessive losses of nitrogen (N) to the surrounding environment. Such events have been found to be responsible for up to ≈70% of the annual soil N loss through NO_3 leaching to groundwater and N_2O emissions (Congreves et al., 2018; Maljanen et al., 2004; Regina et al., 2004; Ryan et al., 2000; Song et al., 2017; van Bochove et al., 2001; Wagner-Riddle et al., 2017, 2008). At the same time the N_2O concentration in the atmosphere is increasing by 0.8 ppb per year (Rochette et al., 2000; Solomon & IPCC, 2007; Wagner-Riddle & Thurtell, 1998). With a ≈120 year atmospheric lifetime, N_2O in the atmosphere has a global warming potential of 298 to 310 times greater than CO_2

(Abdalla et al., 2009; Lamers et al., 2007). Additionally, N_2O results in the destruction of stratospheric ozone and hydroxyl radicals that decrease incoming UV radiation and remove other GHGs (Brown et al., 2001; Crutzen, 1970; Delgrosso et al., 2005; Wang et al., 1976).

Soil nitrification and denitrification drive soil N fluxes, including the non-growing season fluxes (Maljanen et al., 2004; Regina et al., 2004; Wagner-Riddle et al., 2008). However, while the exact interrelationships between drivers of nitrification and denitrification, especially during the plant dormant period, are not well understood, the use of N-cycling models has been proposed to describe the complexity and variability of factors governing soil N forms in agricultural systems (Bell et al., 2012; Bittman & Hunt, 2013; Chambers et al., 1999; Shaffer et al., 2001). Still, much less is known about the effects of FTCs on Atlantic Boreal Maritime agriculture practices, since much of the literature (Bell et al., 2012; Bittman & Hunt, 2013; Chambers et al., 1999; Shaffer et al., 2001) focuses on temperate climate agriculture systems. Studying soil N-cycling in the Atlantic Boreal Maritime is crucial given the anticipated northward shift of agricultural climate into the Boreal ecozone in the face of rapidly changing global climatic patterns (King et al. 2018). Thus, there is a need to extend our current knowledge of soil N-cycling to include Boreal ecozone agriculture systems.

2 OVERVIEW OF THE MAJOR BIOLOGICAL PATHWAYS INVOLVED IN N-CYCLING

2.1 NITROGEN FIXERS AND NITROGEN-FIXING GENES

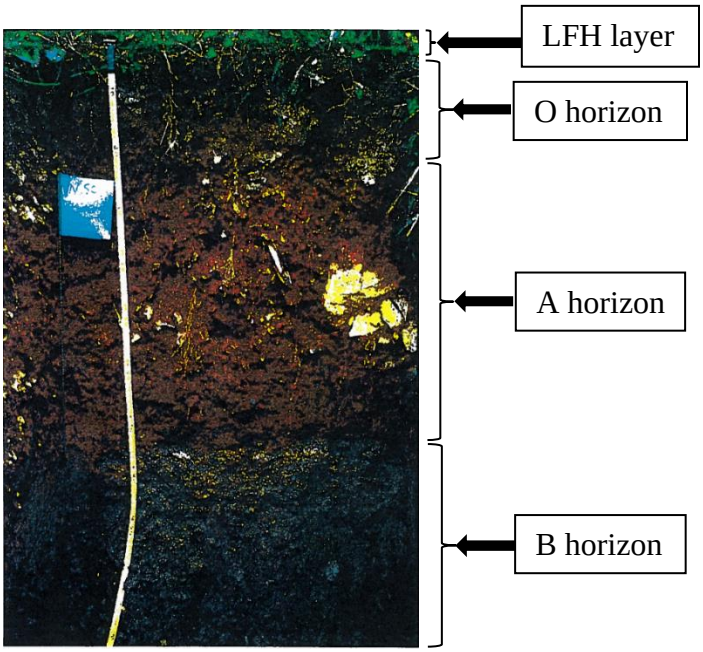

Figure 2.1. *Typical appearance of the soil horizons* - *the litter, folic and hummus (LFH), mineral (O) and subsoil (A) horizons)* - *for the Cochrane series (Woodrow et al., 1996).*

On non-managed lands soil nitrogen processes primarily occur in the carbon-rich upper layers of soil (**Figure 2.1**) and are regulated by microorganisms, whose activities vary spatially and temporally (Butterbach-Bahl et al., 2013; Congreves et al., 2018; Giles et al., 2012; Groffman et al., 2009; Pajares & Bohannan, 2016; Philippot et al., 2009; Shaffer et al., 2001; Shaffer et al., 2001; van Groenigen et al., 2015). Biological nitrogen fixation (BNF) is an energetically expensive process converting atmospheric dinitrogen (N_2) into R-NH_2. It is limited to the prokaryotes of the Archaea and Bacteria domains,

and is catalyzed by the *nifH* gene-encoded nitrogenase reductase (Robertson & Groffman, 2015).

2.2 NITRIFIERS AND NITRIFYING GENES

Nitrification is the aerobic oxidation of ammonium to nitrate via nitrite (**Figure 2.2**; Bitton, 2002; Paul, 2007). Although, a variety of bacteria, archaea and fungi are able to carry out nitrification, chemolithoautotrophic nitrifiers (ammonia-oxidizing bacteria [AOB], archaeal ammonia oxidizers [AOA] and nitrite oxidizing bacteria [NOB]) and codenitrifying fungi are the dominant group of microorganisms that facilitate the nitrifying processes in most soil ecosystems (Braker & Conrad, 2011; Hayatsu et al., 2008; Hora & Iyengar, 1960; Hu et al., 2015; Li et al., 2017; Long et al., 2013). As seen in **Figure 2.2,** nitrification begins with ammonia oxidation to hydroxylamine ($NH_4^+ \rightarrow NH_2OH$) which is performed by the AOB and AOA groups that produce the enzyme ammonia monooxygenase (AMO) encoded by the *amoA* gene through the *amoABC* operons (Braker & Conrad, 2011; Hu et al., 2015). The second step catalyzes hydroxylamine to nitrite ($NH_2OH \rightarrow NO_2^-$) by a unique enzyme belonging to AOB, the *hao* gene-encoded enzyme hydroxylamine oxidoreductase (HAO). Finally, the last step ($NO_2^- \rightarrow NO_3^-$) is regulated by the NOB via the *nxrB* gene-encoded enzyme nitrite oxidoreductase (NXR; Braker & Conrad, 2011; Hu et al., 2015; Xi et al., 2016).

Additional processes includes the anaerobic ammonium oxidation (anammox) coupled with dissimilatory nitrate reduction to ammonium (anammox and DNRA in **Figure 2.2;** Pajares & Bohannan, 2016; Reed et al., 2014; Ward, 2013; Xi et al., 2016).

Of the two, DNRA has been well studied in various conditions, but both reactions have not yet been assessed in boreal forest agricultural production systems.

2.3 DENITRIFIERS AND DENITRIFYING GENES

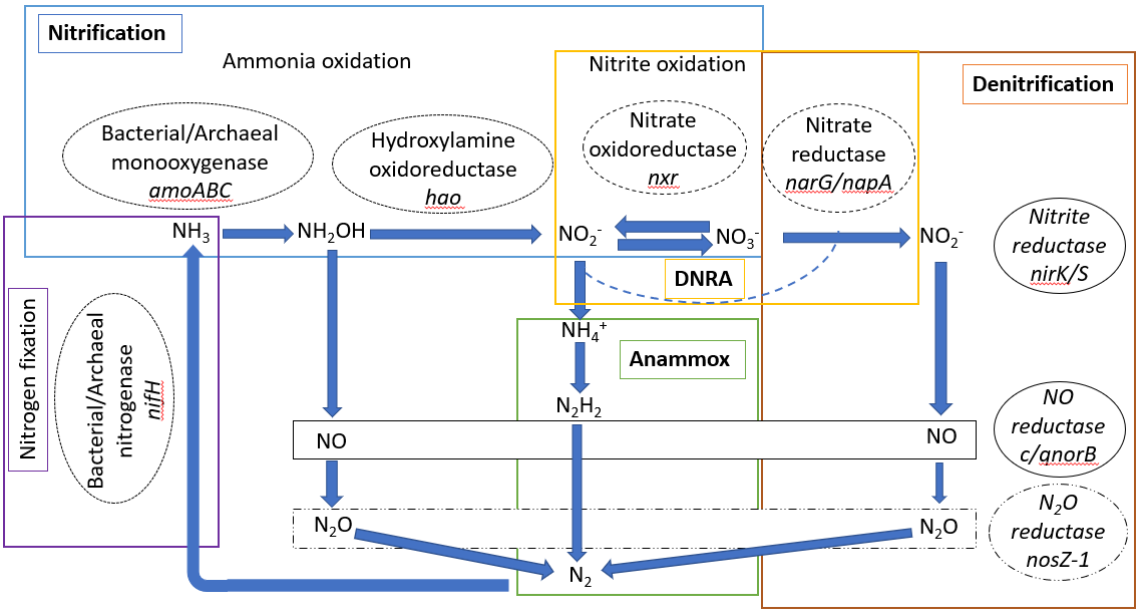

Figure 2.2. *The soil nitrogen cycle with associated genes and enzymes.*

Denitrification is the stepwise reduction of NO_3^- to several gaseous end products (**Figure 2.2**; Bitton, 2002). The process involves a diverse bacterial taxa of *Proteobacteria*, *Firmicutes*, *Actinomycetes*, *Bacteriodetes* and A*quificeae*, and as well as fungi (Braker & Conrad, 2011; Long et al., 2013; Philippot et al., 2007). However, denitrification has a polyphyletic origin, thus it is nearly impossible to apply a 16S rRNA gene-based approach (a standard taxonomic technique used more generally for bacterial identification) to the study of the primary taxa most heavily involved in denitrification; thus a large portion of literature is based on targeting functional bacterial marker genes (Braker & Conrad, 2011). The first step of bacterial denitrification (NO_3^- → NO_2^-) –

which is mediated by both denitrifiers and nitrate respirers – involves nitrate reductase encoded by the *narGH* and *napA* genes; then, two different nitrite reductases encoded by the *nirK* and nir*S* genes perform the second step (NO_2^- → NO). The third step (NO → N_2O) is catalyzed by the nitric oxide reductase enzyme encoded by the *cnorB* and *qnorB* genes; the final step (N_2O → N_2) is mediated through the nitrous oxide reductase encoded by the *nosZ* group of genes (Braker & Conrad, 2011; Hu et al., 2015).

2.4 FACTORS CONTROLLING SOIL N FLUXES, AND GENETIC AND FUNCTIONAL DIVERSITY

Slowing the growth or activity of nitrifiers, achieved by amending soils with nitrification inhibitors, is an attractive management option as it keeps soil N in the NH_4^+ form, thus preventing its loss from the soil by nitrate leaching and denitrification (Paul, 2007). The factors controlling soil biological nitrogen-fixing and nitrification and denitrification, which dictate the rate of these processes, are the same included in models to predict the rate of NH_4^+-N, NO_3-N leaching and N_2O production: forage and fertilizer sources effects on soil NO_3^- and NH_4^+, temperature and FTCs, soil N and carbon (C) supply, pH, O_2 supply and the water-filled pore space (WFPS).

2.4.1 *Forage and Fertilizer Sources*

Crops and the addition of fertilizer alter the soil NH_4^+ and NO_3^- supply. For example, in an overwinter soil mesocosm experiment the mid-winter additions of $K^{15}NO_3$ to the 0 to 5 cm surface layer and 12 to 17 cm deep layer led to significantly higher NO_3^- concentrations at the end of the winter compared to the no fertilizer control (Wagner-Riddle et al., 2008). Moreover, the ^{15}N tracer study of the same experiment found that

NO_3^- concentrations in the adjacent layers of the experimental treatments were significantly higher compared to the adjacent layers in the no fertilizer control treatments; suggesting a vertical movement of NO_3^- in the soil column. Tatti et al. (2014) found a similar trend showing that time and the N source had a significant effect on soil NH_4^+ concentrations over winter, which were generally higher in treatments using poultry manure over cattle manure or mineral fertilizer; however time, rather than the N sources, was more influential on soil NO_3^- concentrations which peaked near the end of winter. A short term experiment studying the effects of mineral or organic fertilizer on long-term amended soils found that mineral fertilization and urban organic waste compost management systems that received mineral fertilizer (NH_4NO_3) had increased soil NO_3^- concentrations (Tatti et al., 2013). The addition of compost or mineral fertilizer significantly increased soil NH_4^+ concentrations in both conventional and organic management systems. Type of crop residues, do have distinct effects: incubations with barley straw (*Hordeum vulgare* L.) (250 mg C kg^{-1} soil) and KNO_3-N (50 mg kg^{-1} soil) led to higher overall concentrations of soil NO_3^- throughout the incubation period versus similar incubations with red clover residue (*Trifolium pratense* L.) (Miller et al., 2008). Soil NH_4^+ concentrations in the same experiment significantly increased over time for both the barley straw (i.e., from 3.0 to 9.6 NH_4^+-N kg^{-1} soil) and the red clover residue treatments (from 5.0 to 9.3 NH_4^+-N kg^{-1} soil). Incorporation of composted dairy manure, in an organic wheat trial, led to increased NH_4^+ concentrations later in the growing season and during the early spring thaw; same fertilisation in an parallel organic alfalfa trial, led to increased NO_3^- concentrations during the fall and spring thaw (Westphal et al., 2018). Another crop trial showed that soils in a potato-red clover crop rotation had greater

concentrations of NO_3^- compared to soils in a potato-barley crop rotation. For both rotations the NO_3^- concentrations were largest at the end of the winter (Tatti et al., 2017). However, the differences between the two rotational treatments were not consistent for the two winters during the trial; for the second winter there was no significant difference in the NO_3^- concentrations. One explanation given for the increase of bioavailable N was the deacclimation of plants during winter warm spells that are followed frost damages to the roots of plants and their subsequent breakdown (Kreyling et al., 2015). A reduced snow cover, expected with the current climatic patterns and winter warm spells, may become important drivers for the winter mineralization and availability of labile organic C and bioavailable N for soil nitrifiers and denitrifiers (Campbell et al., 2014; Kreyling et al., 2015; Wipf et al., 2015; Zhang et al., 2014) , which in turn may affect the winter soil bioavailable N and leaching rates.

Tatti et al. (2013) have shown that the abundance of *nirS*, *nirk* and *nosZ* genes and *nosZ* transcripts were generally higher in urban organic waste compost treatments than in conventional, mineral fertiliser treatments. On the other hand the abundance of *nirS*, *nxrA*, *amoA* AOA and *amoA* AOA transcripts levels did not change among N sources, with the exception of *nirK* gene which was more abundant in soils fertilized with poulty manure (mean of 9.3×10^6 copies g^{-1} dry soil) compared to mineral fertilizer and cattle manure (mean of 3.8×10^6 and 4.2×10^6, respectively) (Tatti et al., 2014). Nevertheless this was not consistent for a subsequent winter when the *nirS* genes were significantly more abundant in poultry manure treatments (mean of 8.16×10^6) compared to the mineral fertilizer (mean of 3.84×10^6); the abundance of *nirK* genes was greater in poulty

manure (mean of 3.3×10^7) compared to mineral fertilizer and cattle manure (mean of 9.1×10^6 and 1.01×10^6) treatments respectively (Tatti et al., 2014). The same study found that the winter abundance of *Nitrobacter*-like *nxrA* genes was significantly affected by N sources; they were more abundant in cattle manure (mean of 4.5×10^7) than in poultry manure or mineral fertilizer treatments (mean of 2.1 and 1.5×10^7, respectively).

Similarly, inconsistencies in functional nitrogen cycle related gene expression were also found out in other trials. While Tatti et al. (2017) found that in the winter of 2010 for example, while in one winter the abundance of *nirS, nirk* and *nrfA* nitrite ammonifiers were the same in both potato-barley and potato-red clover treatments (mean of 1.12×10^7, 1.22×10^6 and 4.86×10^6 copies g^{-1} dry soil respectively), for the subsequent winter the abundance of *nirS*, *nirk* and *nrfA* nitrite ammonifiers were significantly greater in soils of the potato-barley treatment (mean of 2.1×10^6, 9.3×10^5, 3.3×10^6 copies g^{-1} dry soil respectively) compared to the potato-red clover treatment (mean of 0.9×10^6, 4.2×10^5, 8.5×10^5 copies g^{-1} dry soil respectively). These studies show that during the winter forage crops and fertilizers in agricultural soils act as sources of bioavailable N and labile organic C that facilitate microbial growth, and thus both affect soil nitrifier and denitrifier gene abundance and expression. Although there is some information on the effect of crop and fertilizer sources on nitrification and denitrification functional genes, no information is available on the winter effect of crops and fertilizer on *nifH* gene abundance or expression.

2.4.2 *Temperature and Freeze-Thaw Cycles*

The cold season in the boreal climate or ecosystem (late September – late March) can be divided into three phases: a gradual decrease towards 0 °C with small periods of freeze-thaw, a prolonged frozen period and, finally, recurring cycles of freeze-thaw commonly including the melting of frozen soils. The intensity and length of each period is subject to geographical factors (longitude and latitude), local weather and yearly climatic fluctuations (Öquist et al., 2004). Microbial activity has been recently identified to occur at sub-zero temperatures in high latitude areas (Philippot et al., 2007; Sorensen et al., 2018; Ouyang et al., 2017; Wertz et al., 2016, 2013; Tatti et al., 2017, 2014; Sharma et al., 2006; Bittman & Hunt 2013; Öquist et al., 2004; Wagner-Riddle et al., 2010, 2008). Parallel studies have found that this microbial activity may be responsible for up to 70% of annual N_2O production during FTCs (Maljanen et al., 2004; Öquist et al., 2004; Teepe, Brumme, & Beese, 2001; Wagner-Riddle et al., 2008). Of more concern are temperate and cropland ecosystems since a meta-analysis by Gao et al. (2018) proposed that FTCs significantly increased soil NO_3^-, NO_3^- leaching and N_2O emission by 18.3%, 66.9 % and 144.9% respectively.

There have been many suggested explanations for this increase in NO_3^- or N_2O production. Kreyling et al. (2015) and Campbell et al. (2014) have suggested that ≥82% of the N produced during respective FTCs studies was from biological origin before it was leached from the soil. Gao et al. (2018) and DeLuca et al. (1992) suspected that freeze-thaw events disrupt soil aggregates and microbial cells since the release of mineral N was directly related to soil microbial biomass. The release of nutrients and increased

temperatures during thaw are thought to accelerate nitrifier activities and facilitate the transformation of NH_4^+ to NO_3^- (Gao et al., 2018). Ludwig et al. (2004) for instance saw 5.67 mg of NO_3^- (kg $soil^{-1}$) d^{-1} produced during 5 days (d) of thawing (at 8 °C) after 7 days of freezing (at -7 °C). Similarly, Mørkved et al. (2006) found that when oxygen was not limiting, freeze-thaw-treated soil produced 1.5–1.8 μg NO_3-N g^{-1} dry weight (dw) soil d^{-1}. The number of snowmelt events are thought to increase in the winter with more frequent and intense freeze-thaw cycles; the resulting phenomena leads to the removal of newly produced NO_3^- and labile organic carbon from the soil via large volumes of water that percolate through the soil column (Campbell et al., 2014; Wipf et al., 2015). Joseph and Henry (2008) saw similar trends and found that total NO_3^- leached approximately tripled between control and FTCs soil core treatments (≈300 and ≈800 μmol m^{-2}) in response to as little as two FTCs, with great losses sustained after repeated (i.e. >7) FTCs.

Previously, several reports (Burton & Beauchamp, 1994; Maljanen et al., 2009; van Bochove et al., 2001) suggested that N_2O production in the unfrozen subsoil is unable to diffuse through frozen upper soil surfaces and may account for the initial burst of N_2O during spring thaw. However, Wagner-Riddle et al. (2008) reported that the source of the N_2O burst at the surface layer during spring thaw was mostly newly produced N_2O. It has been speculated that part of the microbial cells are killed during FTCs, releasing nutrients for surviving microbes, including denitrifiers (Congreves et al., 2018; Ryan et al., 2000). Teepe et al. (2001) and Congreves et al. (2018) have shown that denitrifying microorganisms were promoted during continuous soil freezing due to an unfrozen water film being formed in the soil matrix limiting oxygen supply between soil particles.

Studies like Tatti et al. (2014) found that N_2O emissions were low (0.34 mg N_2O-N ha^{-1} d^{-1}) at temperatures around 0.5 °C during December but experienced a 100-fold increase (34.6 mg N_2O-N ha^{-1} d^{-1}) when temperatures increased to about 3 °C in the following January. Sharma et al. (2006) found that cores exposed to one day of freezing at -20 °C had produced approximately 1 mg N_2O-N kg^{-1} soil after 1 day of thawing, which gradually increased and peaked at 6.2 mg N_2O-N kg^{-1} soil after 5 days of thawing. For Wertz et al. (2016), soil cores with N amendments that were frozen for 3 days at -5 °C produced approximately 10 to 15 μg N_2O-N kg^{-1} dry soil $hour^{-1}$ respectively after 1 day of thaw at +4 or 15 °C; those cores exposed to a thaw at 15 °C peaked at approximately 20 μg N_2O-N kg^{-1} dry soil $hour^{-1}$ after 2 days of thawing. These same soils experienced an overall decrease in N_2O production after 4 days. As the duration, amplitude and frequency of FTCs increases in the current global climatic pattern, it is expected to result in a larger release of N_2O emissions from boreal agricultural soils (Gao et al. 2018).

It has been suggested that most free-living diazotrophs are mesophilic and typically prefer temperatures between 15 to 35 °C (Bitton, 2002). Several field studies have investigated the *nifH* gene abundance in cold climates (Penton et al., 2016; Wang et al., 2016; Yergeau et al., 2007; Yergeau & Kowalchuk, 2008). A summer and winter alpine grassland mesocosm experiment showed that *nifH* gene abundance declined during a climate change simulation, i.e. when soil cores from a high elevation site were transported to a low elevation site and left to acclimate over summer and winter by (Wang et al. 2016). Similarly results of a laboratory microcosm study suggests that *nifH* gene expression may be positively or negatively influenced by the increasing frequency

of FTCs (Yergeau & Kowalchuk 2008). The activity of gene of N-fixers in these two climate change studies are likely associated to free-living organisms as opposed to plant-associated N-fixers, since the latter will only fix N_2 using labile C in the rhizosphere zone of an actively growing plant (Paul, 2007). Although there are cold climate field studies investigating the *nifH* gene abundance there are no winter field studies on the *nifH* gene expression in the Canadian temperate and/or boreal context.

Sharma et al. (2006) were one of the first research teams to document the effects of FTCs on nitrifying and denitrifying gene abundances. Their study reported, after a 1 day freeze at -20 °C and subsequent thawing at 10 °C for 9 days, that at 2 days there was a 5- and 10- fold increase in *napA* and *nirS* transcripts; however transcript numbers decreased after the 3rd and 9th day. Tatti et al. (2015) reported that *nirK*, *nirS* and *nosZ* gene abundance was lowest during the FTCs winter months (i.e., December and January) and slowly increased when the soil temperature stabilized near 0 °C (February) or increased above 0 °C (March). Wertz et al. (2016) had similar results with denitrification gene abundance; there was an increase in *nirK* and *nirS* abundance (gene copies g^{-1} dry soil) from day 0 to day 7 (after 3 days at -5 °C followed by either 7 days at either +4 or +15 °C) in N amended soils. Additionally, soil thawing at +15 °C had higher *nirK* and *nirS* gene abundance than soils thawing at +4 °C. Similarly, Tatti et al. (2014) determined that nitrifier (*Nitrobacter*-like *nxrA* and archaeal *amoA*) and denitrifier (*nirK* and *nirS*) abundances were lowest (between 3.6×10^6 and 1.1×10^7 copies g^{-1} dry $soil^{-1}$) during the FTCs months (i.e., November and December) but steadily increased (from 2.8×10^6 to 7.76×10^6 copies g^{-1} dry $soil^{-1}$) in the warmer March and April months. Isobe et al. (2018)

described comparable trends in nitrifier (AOA-*amoA*) and denitrifier (*nirK* and *nirS*) gene abundances, though AOB-*amoA* abundance had opposite trends and had a comparably higher abundance during the mid-winter months (December and February). As inferred by Tatti et al. (2015) and Su et al., (2010), frequent freeze-thaw cycles may reduce the abundance of nitrifying (AOB-*amoA*) and denitrifying genes (*nirK*, *nirS* and *nosZ*) due to frost induced lysis of part of these communities due to persistently absent snow cover during the winter season.

2.4.3 *Soil aerobiosis as a function of soil pores saturation status*

Soil water content controls various soil abiotic parameters including soil oxygen levels and pH; and favours transports and diffusion of reactants to and from site of reactions (Paul, 2007). Since nitrogenase is highly sensitive to denaturation by O_2, studies have found that rates of biological nitrogen fixation can increase significantly in anaerobic conditions and is optimum at 2% $O_{2;}$ making soil moisture an important factor for biological nitrogen fixation (Hicks et al., 2003; Pajares & Bohannan, 2016; Paul, 2007). Nitrification is favoured when there is a steady supply of molecular oxygen at ≤ 60% water-filled pore space. Denitrification is the main process that occurs under low oxygen levels, i.e. when water-filled pore space of the soils is above 60% (Paul, 2007; Bitton, 2002; Robertson & Groffman, 2015). Sexstone et al. (1985) confirmed this experimentally when they found that the centers of soil aggregates are surrounded by a thin film of water that impedes efficient gas exchange, owing to a respiratory demand in the aggregate higher than the gas diffusion rate. However, denitrifiers are facultative anaerobes that do not produce denitrification enzymes until O_2 is low or depleted (Paul,

2007). When oxygen is limiting, denitrifiers use nitrite, or any N-intermediate supplied from nitrification or denitrification, as an electron acceptor for respiration (Hu et al., 2015; Paul, 2007)

Several studies have found that the flush of soil water during FTCs and at the end of the winter season (March and April) may result in a adequate or restricted O_2 supply and thus favourable conditions for cold-adapted nitrifier and denitrifiers gene expression (Németh et al., 2014; Tatti et al., 2015, 2017; Wertz et al., 2016). Though there are no current studies on the effect of soil water-filled pore space or volumetric water content on *nifH* gene transcript abundance, it can be expected that during an increase soil water-filled pore space or volumetric water content (VWC) there is a simultaneous increase in the transcription of *nifH*, *nirK*, *nirS* and *nosZ* genes. However, the expression of nitrifying genes (*Bacteria*- and *Archaea-amoA*) may be related more so to the flush of nutrients found in the soil water with the results varying depending on the time of year.

2.4.4 ***Soil Organic Carbon and Nitrogen Supply***

Next to photoautotrophs, free-living N-fixers are the second most important biological organisms for continuously fixing N_2 into NH_3 and thus supporting high rates of plant primary production (Bitton, 2002). However, the activity of free-living N-fixers has been suggested to be inhibited by an increase in bioavailable N, e.g. NH_4^+ or NO_3^- (Penton et al., 2016). Down-regulation of the *nifH* gene expression is related to increased levels of soil NO_3^-, NH_4^+ and/or amino acid N (Paul, 2007). This ‘switch-off’ plays an important role for N-fixers as it allows growth to continue in the presence of an bioavailable nitrogen source, e.g. NO_3^-, NH_4^+ and/or amino acid N (Kessler et al., 2001).

Another key source of N in soil is the biologically mediated oxidation of soil organic C (SOC). Since biological nitrogen fixation is an energy intensive reaction, it takes 2,054 kg of C to fix 45.36 kg of N_2. Thus, as organic carbon becomes more plentiful so does the rate of N-fixation (Bitton, 2002). Consequently, during FTCs large quantities of labile dissolved organic C and bioavailable N are mobilized (Campbell et al., 2014; Tatti et al., 2014). It can thus be inferred that during the non-growing season an increase in bioavailable N and C in soil water flushed from FTCs may stimulate N-fixers but down-regulate *nifH* gene expression. It has been shown that N-fixation rates is significantly and positively correlated to *nifH* gene abundance, soil organic C, TN; but *nifH* gene abundance was positively correlated to soil organic C, dissolved organic C, and TN and negatively correlated to bioavailable N supply (Chen et al., 2019; Keshri et al., 2015; Liu et al., 2019; Wang et al., 2017; Wang et al., 2017). At least in one report, *nifH* gene abundance was found to be positively correlated to bioavailable N (Chen et al., 2019). Still, these studies suggest that, given the high energy demand of N-fixation, N-fixers will preferentially assimilate the increased soil organic C and bioavailable N (Pereira e Silva et al., 2013) to continue microbial growth, resulting in the down-regulation of the *nifH* gene expression.

The ammonium (or NH_4^+) supply is usually the most important factor controlling the rate of nitrification (Bitton, 2002; Li et al., 2017; Paul, 2007; Tourna et al., 2008). Nitrifiers are poor competitors for soil NH_4^+ and are often outcompeted by the high plant/heterotroph demand for N; thus nitrification rates are usually low when high Carbon:Nitrogen (C:N) ratio-residues are added to agricultural soils (Paul, 2007). If there

are no other limiting factors, nitrification usually accelerate when fertilization exceeds the high plant and heterotroph uptake of soil NH_4^+ (Robertson & Groffman, 2015). Similarly, denitrification is also controlled by soil organic C and NO_3 since most denitrifiers under anoxic conditions require reduced C as an electron donor. However, the relative influence of the soil C and N supply as major players depends on the ecosystem (Paul, 2007). For example, an abundance of soil C, in aerobic soils (i.e., water potential <60%) limits the rate of denitrification as it provides denitrifiers, being facultative anaerobes, with oxygen as a final electron acceptor (Paul, 2007).

Isobe et al. (2018) reported that AOB-*amoA* gene abundance was positively correlated to soil NO_3^- and NO_2^- levels (r = 0.40 and 0.37 respectively), while AOA-*amoA* were somewhat negatively correlated to these soil N concentrations (r = -0.17 and -0.09 respectively). Qin et al. (2018) reported that the N_2O flux rate was positively correlated to active and present *nirS* abundance, while the AOB-*amoA*, and *nosZ* gene abundances were correlated to high concentrations of soil NH_4^+ and dissolved organic carbon. Samad et al. (2016) concluded that the N_2O:N_2 emissions ratio (i.e., the proportion N_2O produced to total emissions [N_2]) was highly negatively dependent (r^2 of 0.57 and 0.63) on the total denitrification gene abundance (*nirK, nirS, nosZ-I and nosZ-II*). In other words, the N_2O:N_2 decreases with an increase in total denitrification gene abundance. Tatti et al. (2015, 2017) suggested that the abundance and expression of denitrifying genes responded to an increase in NH_4^+ concentrations under both studies. Similarly, nitrifying (AOA- and AOB-*amoA*) and denitrifying (*nirK* and *nirS*) gene abundance increased with increasing NO_3^--and NH_4^+-N, soil organic C and water

extractable organic carbon (Hai et al., 2009; Ouyang et al, 2016; Su et al., 2010). Moreover, studies have correlated the changes in the AOA- and AOB-*amoA* gene abundances and microbial community profiles to potential nitrification rates and the paired carbon and nitrogen metabolism, i.e. the oxidation of large quantities of ammonia to fuel small amounts of carbon fixation (Norman et al, 2015; Ouyang et al., 2017, 2016). The results of these investigations suggest that both nitrification and denitrification are tightly coupled; and that the soil C and N supply has an influence on nitrification, which in turn influences denitrification. The inherent activity of these functional microbial communities have the potential to stabilize NH_4^+- and NO_3^--N availability, through the upregulation of *amoA-hao* mediated nitrification pathway (Lamba et al., 2017). Even if a lag is seen in the response of the gene abundance profiles of nitrifiers and denitrifiers to the soil N and C supply, possibly due to the slow metabolic response of the enzymes involved in soil N, it is still possible to infer that the expression of functional genes involved in N-cycling are induced by soil N and C supplies.

2.4.5 ***Soil pH***

Soil pH is the key parameter governing chemical and biochemical processes in the soil. Likewise, pH affects the soil N-cycling microbes and therefore soil functions (Paul, 2007). Fertilizer N and N speciation along the nitrification pathway may affect soil acidity itself. Reducing NH_4^+ to NO_3^- frees H^+ ions and thus a decrease in soil pH (Paul, 2007). Free-living nitrogen-fixing bacteria prefer soils with pH ranging from 6.8 to 7.2 but may also thrive in more acidic or slightly basic soils (<6.8 and 7.5 to 8.5; Bitton, 2002). Until recently, it was assumed that nitrifiers are inhibited in acidic soils as cultured

bacterial ammonia oxidizers did not grow in media with pH <5.5, leading to the belief that nitrification is also inhibited (De Boer & Kowalchuk, 2001; Li et al., 2017). However, despite an optimum pH range between 7.5-8 (Paul, 2007), nitrification was found to occur in exceptionally acidic environments (pH <4.5) (Li et al., 2017; Norton & Stark, 2011; Paul, 2007).

While several studies have found that *nifH* gene abundance is affected by and/or negatively correlated to increasing soil pH (Chen et al., 2019; Keshri et al., 2015; Pereira e Silva et al., 2013; Su et al., 2010; Honglei Wang et al., 2017), there is still a knowledge gap on the over-winter effect of pH on *nifH* gene transcriptional activity. In acidic soils, ammonia-oxidizing archaea and their transcriptional activities are found to be functional and speculated to be responsible for the nitrification in these soils (Li et al., 2017; Nicol et al., 2008; Sun et al., 2015). When the soil pH increases from 4.9-7.5, the transcriptional activity (transcript copies g^{-1} dry $soil^{-1}$) of AOA-*amoA* is reduced but, in contrast, the transcriptional activity of AOB-*amoA* increases (Nicol et al., 2008). Ouyang et al. (2016) showed a somewhat homologous trend but found that AOB-*amoA* gene abundances were more affected by changes in soil pH than AOA-*amoA*, suggesting that the latter is more resistant to ambient disturbances brought upon by soil management practices, e.g. tillage or fertilization. Guo et al. (2017) confirmed significant positive correlations between the AOA and AOB gene abundance with soil pH (r=0.35 and 0.8 respectively). Research on the effects of soil pH on denitrifying gene activity is limited, but a study by Samad et al. (2016) found a strong positive relationship (r^2=0.46) between increasing soil pH and total denitrification (*nirK, nirS, nosZI and nosZII*) gene abundance. A similar investigation by

Sun et al. (2015) found that soil pH was a significant factor controlling the abundance of *nirK*, *nirS* and *Bacteria-amoA* functional genes. Previously, Tsiknia et al. (2015) and Guo et al. (2017) found results similar to the latter study, i.e., significant positive correlations to soil pH with the denitrifying gene chains, but also including *nosZ* clades I and II.

2.5 THE USE OF N-CYCLING MODELS

The nitrification and denitrification processes (**Figure 2.2**) have been studied extensively, concluding that they are the primary sources of N_2O gaseous loss and NO_3-N leaching following the application of mineral or organic N fertilizers (Plaza-Bonilla et al., 2014). To approximate a detailed model simulation, an all-inclusive list of parameter inputs is generally required (Ritchie & Dent, 1994). DeNitrification-DeComposition (DNDC; Li, et al., 2000, 1992b, 1992a; Norman et al., 2008; Stange et al., 2000) and Nitrate Leaching and Economics Analysis on Stella (NLOS; Hirsch, 2007; Shaffer et al., 2001) are examples of programs that are developed for agricultural soils in the Canadian temperate climate.

2.5.1 *Model comparisons: DNDC and NLOS*

Both models require data on soil moisture content, quantity of N fertilizer or crop residue applied, crop type, soil type and texture, tillage, freeze-thaw events and irrigation as state variables. However, there are dissimilarities between them; for example, NLOS considers daily weather patterns, air temperature, pH and differences in N-production by depth, whereas DNDC does not incorporate these variables. On the other hand, DNDC does include sub-zero air temperatures, soil organic C, biomass and snow coverage, and

precipitation (Abdalla et al., 2009; Li et al., 1992a, 1992b; Thomas et al., 2013; Westphal et al., 2018). In addition, NLOS does not include O_2 concentrations for oxidation, soil temperature, and changes in soil N species between seasons. Furthermore, the function (i.e. a first order equation or similar) used to quantify soil N species is different in each of the models. Of the two, DNDC to date has been well tested in a variety of geographic regions and crop types, but requires calibration and validation studies to cover a wider scope of areas (Thomas et al., 2013). For example, the accumulated N_2O emissions from a spruce forest during FTCs calculated by Norman et al. (2008) found that a modified DNDC model both underestimated (-41%) and overestimated (15%) the field data throughout a 4 month simulation period. At the moment, estimating field soil N transformations in the Canadian boreal agricultural context via models is limited. For more information for the state variables used in each model please view **Table 2.1**.

Table 2.1. *The functional state variables used to quantify and predict the soil N species in the NLOS and DNDC programs. + and × refers to the respective inclusion or exclusion of the state variable.*

State variables	**DNDC**	**NLOS**
Soil moisture content	+	+
N fertilizer/Crop residue (kg)	+	+
Crop type	+	+
Soil type	+	+
Texture	+	+
Tillage	+	+
Freeze-thaw events	+	+
Irrigation	+	+
Daily weather	x	+
Precipitation	+	x
Air temperature	x	+
Soil-N species by depth	x	+
pH	x	+
Sub-zero air temperature	x	+
Soil organic carbon	x	+
Snow depth/coverage	x	+
O_2 cocentrations	+	x
Soil temperature	+	x
Soil-N species by season	+	x
Calibration and validation in several geographic systems	+	x

2.5.2 *Modelling limitations*

It should be noted, as stated by Cabrera et al., (2008), that some models (e.g. DNDC) incorporate a smaller sub-model of the potential soil microbial biomass and population growth driving nitrification and de-nitrification. However, there is disagreement on whether soil microbial biomass and population growth accurately reflect the rate and activity of nitrifying and denitrifying enzymes. For example, a study by Parsons et al. (1991), using the most-probable-number counts, failed to determine a relationship between the denitrification rate of N_2O evolution with the denitrification

population growth and enzymatic activity. Conversely, Drury et al. (1991) found that soil microbial biomass (C) was positively correlated to N_2O denitrification rates (r = 0.854) but said correlation was reduced with potential denitrification assays (r = 0.07) after amendments with soluble C and NO_3^-. Zaman et al. (2006), on the other hand, determined that the nitrification rates were weakly correlated to soil microbial biomass N (r = 0.39) with a lower correlation to microbial biomass C (r = 0.18).

These results suggest that an exhaustive list of state variables is needed to model soil N dynamics but several reports (Mary et al., 1998; Molina & Smith, 1997; Shaffer et al., 2001; Willigen, 1991) disagree with this proposition and have argued that simple functional state variable models (i.e. empirical or stochastic) may perform better than their complex mechanistic counterparts. Quantifying the dynamic behaviour of soil N through modelling is hampered by the heterogeneity across a landscape and it requires intensive field sampling and laboratory analyses to allow effective calibration and validation of model outputs for detailed spatial and temporal field estimates of soil N (Bittman & Hunt, 2013; Butterbach-Bahl et al., 2013). Still, mechanistic or stochastic models based solely on abiotic parameters are bound to require extensive calibration and sensitivity studies in order to be sufficiently robust to be applied practical decision-making. Furthermore, most models were originally developed for mineral soils, and thus fail to correct for ammonia volatilization following land application of livestock manure (Smith, 2005). Moreover given the expected northward shift of agriculture into the Boreal ecozone there is a need for more N-cycle modelling in agriculture fields based in this ecozone (King et al., 2018). Differences in the set of necessary biotic and abiotic

parameters needed to efficiently execute the DNDC and NLOS models and the failure to make accurate predictions under an array of climatic regions may be due to limitations in the absolute quantitation and inclusion of the soil microbial genetic and functional diversity via previously inaccessible molecular techniques at the time of sampling, as well as their biotic and abiotic triggers. Nevertheless, many studies to date (Sharma et al., 2006; Tatti et al., 2015, 2014, 2017; Wagner-Riddle et al., 2008; Wertz et al., 2016) have investigated the N-cycling gene (nitrogen-fixing, nitrification and denitrification in **Figure 2.2**) independently. This offers only a partial understanding of the role of abiotic parameters on gene functions, independent of precursor transcripts.

The information presented in **Section 2.4**, forms the basis of the hypothesis that the production and flux of soil N in acidic, carbon/nitrogen-rich, water-logged soils during the non-growing season in Atlantic Boreal Maritime agricultural systems are correlated to the expression of N-cycling genes. Further, it is hypothesized that the cycling of soil N during the non-growing season can be inferred from the expression and activity of these genes.

2.6 SIGNIFICANCE OF THE RESEARCH: RELATIONSHIPS BETWEEN NON-GROWING SEASON SOIL N, AND N-CYCLE GENE TRANSCRIPT ABUNDANCE AND ACTIVITY

Recent studies have highlighted that a gene-centric approach might more adequately predict the soil N-cycling microbial activity in agricultural systems (Powell et al., 2015; Reed et al., 2014). Models whose goals are to determine the rate of N-fixation and (de)-nitrification have intrinsically quantified the influence of N-cycling genes. Thus, this research aims to determine the value of those genes, when incorporated into a model with biotic and abiotic triggers, to determine the production of soil NO_3-N and NH_4^+-N in an experimental farm field in the Atlantic Boreal Maritime climate.

2.6.1 *Questions*

This project sought to answer several questions:

(1) Which of the N-cycling functional genes (*nifH*, archaea-(AOA) and bacteria (AOB)-*amoA*, *narG*, *napA*, *nirK*, *nirS*, and *nosZ1*) are active during the non-growing season?

(2) What are the abiotic triggers during the non-growing season that are responsible for the shifts in the profile of N-cycle functional genes involved in N-fixing and (de)nitrification?

(3) How do the crop/soil management regimes and changes in soil environmental parameters (soil pH, total carbon, NH_4^+-N, NO_3^--N, and water-filled porosity) during the non-growing season influence the N-cycle functional gene profile in

the Boreal maritime climate? Are there putative relationships among gene expression of different N-cycling functional genes?

a. Specifically, is there any evidence for a correlation between the expression of *nifH*, AOA- and AOB-*amoA* and the expression range of the rest of the N-cycle genes?

(4) How effective is a stochastic model that includes time, abiotic triggers and the (de)nitrifying groups for predicting N-species production during the non-growing season with respect to the crop/soil management?

2.6.2 ***Objectives***

(1) This study quantified gene expression of functional N-cycling genes (*nifH*, AOA- and AOB-*amoA*, *narG*, *napA*, *nirK*, *nirS*, and *nosZ1)* using cDNA and ddPCR in soils amended with whole liquid dairy manure and/or inorganic fertilizer over the non-growing season of 2017-2018.

(2) Possible correlations between changes in functional N-cycling gene expression and changes in soil parameters (soil pH, total carbon, NH_4^+-N, NO_3^--N, and water-filled pore space) were explored.

(3) Models were built using the most informative abiotic and/or biotic parameters to determine the changes in soil NH_4^+-N, NO_3^--N, and functional N-cycling genes.

2.6.3 *Tested Hypotheses*

The features of boreal maritime freeze-thaw cycles in St. John's, NL, Canada lead to several hypotheses about the expression of soil N-cycling functional genes:

(1) Exposure of agricultural soils to cold conditions and FTCs could negatively affect the expression of N-cycling genes.

(2) N-speciation profiles and changes in soil parameters exposed to cold conditions can be attributed to the crop/soil management regimes at specific sampling dates.

    a. Dynamic changes in environmental parameters and N-cycling gene expression profiles are expected in December, March, and June. The soil/crop management regimes typical of Newfoundland agriculture will affect N-cycling gene expression, within these months.

(3) Increased gene expression of N-fixers and denitrifiers is expected over nitrifiers during the FTC period due to the favourable conditions for N-fixation denitrification.

(4) N-cycling gene expression profiles correlate to N-speciation profiles.

    a. Positive correlations exist between N-cycling gene expression and soil concentrations of end products of their respective processes.

(5) N-cycling functional gene expression profiles explain the expression of the different N-cycling functional genes downstream.

3 METHODOLOGY

3.1 SAMPLING LOCATION

The study was conducted on Agriculture and Agri-Food Canada's (AAFC) experimental fields 3 & 12 (at the St. John's Research and Development Centre, Newfoundland and Labrador [NL]). The sites (fields 3 & 12) are part of the Avalon Peninsula of Eastern Newfoundland (**Figure 3.1**). They represent the Maritime Barrens Ecoregion of the Boreal Shield Ecozone (**Figure 3.2**) with predominately Podzolic soils. In the virgin state, these soils were Orthic Humo-Ferric Podzols but since being cultivated and eroded, they are reclassified as Orthic Sombric Brunisols (Heringa, 1981).

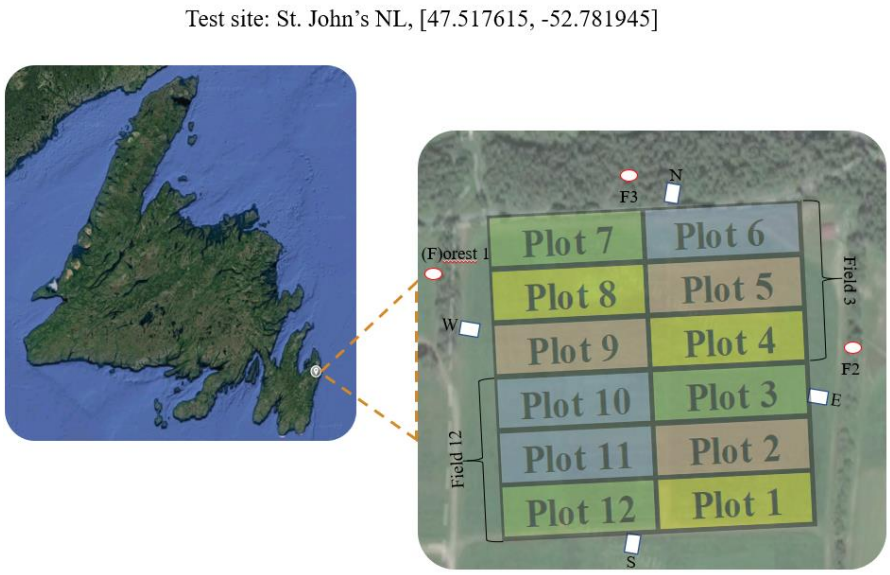

Figure 3.1. *Map of Newfoundland with the St. John's NL, experimental field (47.517615, -52.781945), https://goo.gl/rXD1vG. Colors represent the treatment replicates (n=3 each) for C-G (Conventional Grasses; orange), I-G (Improved Grasses; yellow), C-F (Conventional Forage; blue) and I-F (Improved Forage; green), while red circles with no fill represent the forest (F) control treatment replicates (n=3) for the 2017-2018 (non) growing season* ***Table 3.1****.*

The sites fall within the Atlantic Uplands of Newfoundland, an area of rolling landscapes where glacial till soils are prevalent. The soils at the field sites have developed on acidic, coarse textured, glacial till and are predominately members of the Cochrane series (well drained) with minor areas of the Pouch Cove series (imperfectly drained) (Woodrow et al., 1996). Site slopes range from 2-20% with most slopes being 5-9%. The site is approximately 114 m above mean sea level, with the average daily temperature and precipitation during the non-growing season (October- June) ranging from -5.1 to 11.1 °C and 105.3 to 162.3 mm respectively (Climate Normals data between 1981-2010 accessed from Environment Canada website, https://bit.ly/2UV0Xv6).

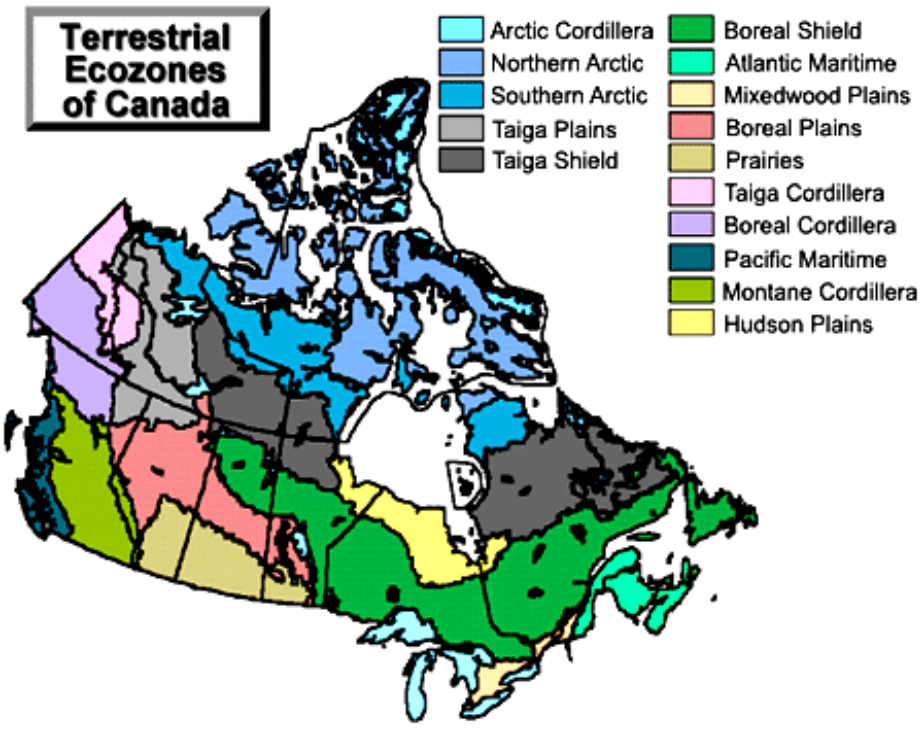

Figure 3.2. *Terrestrial Ecozones and Ecoregions of Canada, https://bit.ly/2PJhGxD.*

3.2 EXPERIMENTAL TREATMENTS

The sites consist of 5.0 ha of land in agricultural use for approximately 80 years; clearing and cultivation began in 1937. Forage crops that were grown in a six-year rotation to this present day are Richmond Timothy (RT), AC Brador Alfalfa (BA), Yukon Tall Fescue (TF), Success Bromegrass (SB) and Preval Meadow Fescue (MF) (**Table 3.1**).

Table 3.1. *Experimental treatments N-V (Natural Vegetation), C-G (Conventional Grasses), I-G (Improved Grasses) and C-F (Conventional Forage) for plots 1-12 amended with liquid dairy manure (LDM) and inorganic fertilizer as nitrogen (N) sources and Forest (F) plots 1-3 with no amendments during the 2017-2018 season.*

ID	Treatment	Crop	N-Source (Fertilizer N is urea based)	Date added	Plot no.
N-V	Natural Vegetation	Mixed-aged Balsam fir (*Abies balsamea*) Red pine (*Pinus resinosa*) and Black spruce (*Picea mariana*)	No fertilizer	-	F1, F2 and F3
C-G	Conventional Grass	Richmond Timothy (*Phleum pretense* ; 8 kg/ha) Preval Meadow Fescue (*Festuca pratensis Huds* ; 8 kg/ha)	Broadcast 10,555 L/plot of whole LDM	May	2, 5 and 9
			24 kg/plot of 46-0-0 fertilizer	July	
I-G	Improved Grass	Richmond Timothy (10 kg/ha) Yukon Tall Fescue (*Festuca arundinacea* ; 1 kg/ha) Success Bromegrass (*Bromus inermis Leyss. x Bromus riparius Rehm* ; 3.5 kg/ha) AC Brador Alfafa (*Medicago sativa* ; 8 kg/ha)	Surface applied 10,550 L/plot of whole LDM	May	1,4 and 8
			57.6 kg/plot of 21-6-18 fertilizer	July	
			Additional 48 kg/plot of 21-6-18 fertilizer	September	
C-F	Conventional Forage	AC Brador Alfafa only; 18 kg/ha	Broadcast 10,555 L/plot of whole LDM	May only	6, 10 and 11
I-F	Improved Forage	AC Brador Alfafa only; 18 kg/ha	48 kg/plot of 5-10-40 fertilizer	May	3, 7 and 12
			38.4 kg/plot of 5-10-30 fertilizer	July	
			Additional 38.4 kg/plot of 0-0-52 + 0.3 B + 6% S fertilizer	September	

Fields 3 and 12 were divided into 12 plots measuring 32 × 60 m each. Each treatment was triplicated using an incomplete Latin Square design. Treatments consisted of varying combinations of crops and were amended with 100 kg of N as recommended by the provincial soil laboratory (**Table 3.1** and **Table 3.2**). RT, TF, SB and MF were seeded in the previous 2016 growing season and, due to the absence of winter kill, were not reseeded in the 2017-2018 (non)-growing season. However, treatments that were seeded with BA in the previous growing season (2016) were reseeded on May 30th, 2017 due to winter kill. N-V consisted of a natural vegetation of mix-aged Balsam Fir, Red Pine and Black Spruce with no fertilizer additions. The conventional grass (C-G) treatment plots consisted of 8 kg ha^{-1} of RT and MF, and in 2017 received broadcasted whole liquid dairy manure (LDM) in early May and an additional 24 kg of 46-0-0 inorganic fertilizer per plot in early July. The conventional forage (C-F) treatment plots consisted of 18 kg ha^{-1} of pure BA and were amended once with broadcasted whole LDM in early May of 2017. The conventional grass/forage treatments used whole LDM with no attempt to match the nutrient requirements of the crop and was used as proxy to replicate the standard practices of the NL agriculture. The improved grasses (I-G) treatment plots consisted of RT, TF, SB and BA at 10, 1, 3.5 and 8 kg ha^{-1} respectively. In 2017 the I-G plots were amended with surface applied whole LDM in early May, 57.6 kg of 21-6-18 inorganic fertilizer per plot in July, and an additional 48 kg of 21-6-18 per plot in early September. The improved grass treatment reflects the mix of plant varieties that include nitrogen fixers, as a proxy for a quasi-sustainable soil/crop management, also mirroring NL practices. The improved forages (I-F) treatment plots consisted of 18 kg ha^{-1} of pure BA, and in 2017 received 48 kg of 5-10-40 inorganic fertilizer per plot in early May, 38.4

kg of 5-10-40 inorganic fertilizer per plot in early July, and 38.4 kg of 0-0-52 +0.3% B + 6% S in early September (**Table 3.1**). The improved forage treatment uses a mineral mix, as a replacement for whole LDM that attempts to match the nutrient requirements of the plants.

Table 3.2. *Provincial soil laboratory report for fields 3 and 12 for recommended nutrient application for the 2017 growing year.*

Newfoundland Labrador

Government of Newfoundland and Labrador
Department of Fisheries, Forestry and Agrifoods
Agrifoods Development Branch
Land Resource Stewardship Division

Interpreting your Soil Test Report

When you receive your soil test report you will see:

Soil test values of Phosphorus, Potassium, Calcium, Magnesium, Iron, Manganese, Copper, Zinc, Boron, Aluminium, and Sulphur, reported in mg/L. These values tell you what are currently in your soil.

Soil test ratings range from L- to E. This tells you the level of fertility for the specific crop. It is based on historical values which show the relationship between the levels of major crop nutrients in the soil and the levels required by the specific crop in order to achieve optimum yields.

pH is the level of soil acidity or alkalinity of the soil. A pH of 7 is neutral, a value greater than 7 is basic and less than 7 is acidic. Soil in Newfoundland is naturally acidic, in a pH range of 4.5-6. Every crop has a required optimal pH, which can vary from 5.0 to 6.5

LR (lime requirement) – The value given on the soil test report is in tonnes/hectare (t/ha) and is the amount required to raise the pH to the level required by the specific crop. By increasing the pH to the optimum level, applied nutrients will be more available to the crop.

CEC (Cation Exchange Capacity) – is the soils ability to hold and exchange certain nutrients (cations) for plant growth. It is an indicator of soil fertility and is expressed in centimole per kilogram of soil (cmol/kg). CEC is dependent on soil texture and can vary from 4 (very sandy soil) to 200 (very clay soil).

Required Applications of Nitrogen (N), Phosphate (P_2O_5), and Potash (K_2O) are based on crop requirements for these nutrients and on the different soil ratings. These nutrients are integral components for plant establishment and growth;

Nitrogen	- stimulates root growth and development and uptake of other nutrients - stimulates plant productivity
Phosphorus	- stimulates root formation and growth - encourages flower development, pollination and seed formation
Potassium	- increases plant vigour and resistance to certain diseases - enhances quality of flowers, fruits and vegetables by improving flavour and colour and by strengthening stems

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Table 3.3. *Provincial soil laboratory report for assessing the chemical content of the whole liquid dairy manure used as nutrient application for field 3 and 12 for the 2017 growing year.*

Analysis Results (as received basis)	
Dry Matter (%)	7.1
pH	6.8
Total Nitrogen (%)	0.30
Total Phosphorous (%)	0.054
Total Potassium (%)	0.25
Total Calcium (%)	0.24
Total Magnesium (%)	0.055
Total Iron (ppm)	83
Total Manganese (ppm)	24
Total Copper (ppm)	29
Total Zinc (ppm)	41
Total Boron (ppm)	2.6
Total Sodium (ppm)	480
Soluble Salts (mS/cm)	-

Nutrients Equivalency	**kg/ton**	**kg/1000 L**
Nitrogen (N)		**1.0**
Phosphate (P_2O_5)		**0.9**
Potash (K_2O)		**2.6**

Note : 1 kg/tonne = 2 pounds/ton
1 kg/1000 L = 10 pounds/1000 gallons

Interpretation :
Ten (10) thousand liters or tones of the manure would supply **10** kg N, **5** kg P_2O_5 and **26** kg K_2O for the 1st year crop. Deduct fertilizer application rate accordingly.

Mineral N fertilizer was added as urea. Whole LDM was applied at rates of 10,555 L per plot as recommended by the provincial soil laboratory report (**Table 3.3**). Broadcast manure application utilized a Nuhn manure spreader, whereas surface application was done with a Nuhn manure spreader with an in-tank agitation attached to a low emission tool bar with a 5-cm drop hose surface applicator adjusted to 19 cm.

3.3 FIELD COMPOSITE SOIL SAMPLES

The first composite samples (i.e. October of 2017; and 3 random sampling locations pooled into one sample) were collected from each of the plots at two depths, the depth 1 (PL; 0-25 cm) and depth 2 (SL; 25-40 cm), by using a bulk density soil auger (AMS) with a cylindrical core liner measuring 5.08 × 7.62 cm (W × L). During the December to March sampling dates, if snow was present it was removed from three random areas within the treatment plots using a standard shovel, then a DeWalt 25501 hammer drill was used to penetrate the frozen soil PL, and finally a sample was taken with the soil auger; sampling for depth 2 consisted of using the soil auger only. The May and June composite samples were collected from each of the plots at two depths using a regular shovel and soil auger (AMS). Each composite sample represented a plot and acted as a replicate for the assigned treatment (coloured plots and numbers in **Figure 3.1)**. The forest plots samples were collected by soil horizon from three random points (F1-3 in **Figure 3.1**) from the nearby Balsam Fir, Red Pine and Black Spruce-dominated forest. Each soil sample was immediately mixed on site, placed in individual polyethylene bag, and then transported to an adjacent AAFC laboratory in an insulated container. The laboratory was within a short walking distance (approx. 200 meters), thus soil samples for measuring the changes in abiotic parameters were immediately frozen at -20 °C, whereas soil samples for nucleic acid extractions were immediately frozen at -80 °C.

3.4 SAMPLING TIME AND CLIMATIC DATA

Sampling was conducted throughout the 2017-2018 fall, winter and spring seasons; the local weather was carefully monitored during the winter to allow for soil sampling to be timed with freeze-thaw events. This allowed for a description of the seasonal changes of the soil abiotic and biotic variables. Daily air and soil temperatures for each sampling date were captured using Onset Hobo ® Bluetooth data loggers (Model no. MX2202). Air and soil temperature were acquired via data loggers (**Figure 3.3**). Data loggers were attached to 3-meter-long twine, which was attached to a stake and marked with orange fluorescent paint for easy recovery at the end of the sampling year. Plots 1, 10, 4 and 7 had 2 data loggers each, which represented depth 1 (12 cm) and depth 2 (32 cm) — a total of 8 data loggers for fields 3 and 12. Similarly, forest plots 1-3 each received 2 data loggers — 8 data loggers for the forest plots. This allowed for the variation in air and soil temperature within the fields and forests plots to be captured. Data loggers were placed in the plots in December 27th, 2017.

Figure 3.3. *Data logger location for acquiring air, soil plough layer (12 cm) and sub-layer (32 cm) temperature.*

Sampling was carried out on a monthly basis after August 2017 and accounted for post-harvest N pools. For each sampling event, 30 samples (15 plots × 1 composite sampling point × 2 depths) were collected from each of field 3, field 12, and the forest plots. With the exception of November of 2017 and April of 2018, there were a total of 10 soil sampling events. However, to maintain the analytical load within practical confines, a total of 180 soil samples (or 6 sampling events) were considered for the 2017-2018 experimental year. As a result, the research focused on the following 6 sampling events: October (as a baseline representation of when soils were not yet exposed to FTCs) and December (a decrease in air/soil temperature with modest FTCs and no snow) of 2017, and February (fluctuations in air temperature but, under snow cover, soil stabilization

close to or below 0 °C), March (fluctuations in air/soil temperature, and thus periods of snow melt and FTCs), May (modest periods of snowfall, but a gradual increase in air/soil temperature above 0 °C) and June (no snow and air/soil temperatures above 0 °C) of 2018. Soil sampling events of December, February and March for forest plot 15 was not collected due to a persistently deep snowpack (≥2 meters) and/or considerable soil surface frost layer inhibiting access to the soils in this area; the I-F treatment plot 12 was not collected in March due to a soil surface frost layer. This lowered the total number of soil samples from a projected n=180 to an actual n=172.

3.5 STUDY SITE SOIL CHARACTERISTICS: SOIL PHYSICOCHEMICAL ANALYSES

Each soil sample was passed through a 2 mm mesh sieve to remove stones, roots and other organic debris and then stored at 4 °C. Soil bulk density of the investigated soils was calculated using Eq (1) after oven drying the October, 2017 soils from an intact bulk density cores of known volume at 105 °C for 24 h. The resulting measurement was corrected for the coarse fragments' (>2 mm) weight and volume (Gregorich & Carter, 2008).

$$\boldsymbol{D_b = \frac{W_5 - W_2 - W_1 - W_6}{V - V_c}} \qquad \textit{Eq (1)}$$

where W_6 is weight and V_c is the volume of oven-dry soil >2 mm in size, W_1 is the weight of the cylindrical core liner, W_2 is the weight of an empty tin, W_5 is the weight of the oven-dry soil <2 mm in size, and V is the volume of the cylindrical core liner.

The soil gravimetric moisture (GMC) was determined by using the gravimetric method; whereby samples of a known quantity (10 g) were weighed and recorded. The difference in mass, after oven drying at 105 °C for 24 h., was the percent mass difference (Gardner, 1965). The volumetric moisture content (VMC) was determined by Eq (2) following the bulk density measurement correction for coarse fragments in soil samples, Eq (1), and used to determine the field soil water-filled pore space (WFPS).

$$\boldsymbol{\theta = \frac{W_3 - W_2}{W_5 - W_2} \times \frac{D_b}{D_w}} \qquad \textit{Eq (2)}$$

For Eq (2), theta is the VMC, W_3 is the wet weight of the soil <2 mm and W_2 is the weight of an empty tin, W_5 is the weight of the oven-dry soil <2 mm in size, D_b and D_w is the bulk density of the soil corrected for the coarse fragments and density of H_2O, assumed to be 1 g/cm^3.

The field soil WFPS, calculated from Eq (3), at the time of sampling was inferred from mass of water released from a sample with respect to the previously measured intact bulk density core and the VMC (Gregorich & Carter, 2008).

$$\boldsymbol{WFPS = \frac{W_w \Big/ \left(V_t - \frac{W_s}{D_p}\right)}{D_w}} \qquad \textit{Eq (3)}$$

where W_w is the weight of water (g), W_s is the dry weight of soil (g), V_t is the bulk volume of the soil, D_p is the assumed mineral particle density of 2.49 for sand, clay and silt (Brogowski et al., 2014; Gregorich & Carter, 2008; Heringa, 1981), and D_w is defined above in Eq (2).

Soil total porosity was inferred from Eq (4) by using the resulting soil bulk density measurements and D_p from Heringa (1981).

$$S_t = 1 - \frac{D_b}{D_p} \qquad \textit{Eq (4)}$$

Soil pH was determined with a Hanna Instruments combined pH electrode (model no. HI 9812-5N) which was dipped into the supernatant of a mixture of 20 g soil and 40 mL of 0.01 M $CaCl_2$ (Sigma Aldrich®) following shaking for 30 min (160 rpm) (Gregorich & Carter, 2008).

Soil NO_3^-- and NH_4^+-N content in soil extracts from a 2 M KCl (Fisher Scientific) 1:10 soil: solution ratio (Gregorich & Carter, 2008) was determined with a Lachat 8500 series Continuous Flow AutoAnalyzer.

Total nitrogen and total carbon (TN and TC) analyses were carried out on a 2400 Series II CHNS/O Elemental Analyzer (Serial no. 241L1405061) using the CHN mode. Approximately 0.15 grams of composite soil was milled until homogeneous in a Retsch® CryoMill, and then 5 mg of soil was added to Isomass Scientific Inc. issued tin cups. The tin cups with the pre-weighed 5 mg of soil was used for TN and TC analysis. Standards, blanks and K-factor samples were included to flush the machine and to stabilize baseline numbers (Ryu & Tenney, 2005). Baseline numbers for the standards, blanks and K-factor were determined by using Perkin-Elmer issued Acetanilide, with known quantities of C, N and H, as follows: 71.09, 6.71 and 10.36 % respectively.

3.6 BIOLOGICAL ANALYSES

3.6.1 *Soil RNA extraction and cDNA synthesis*

RNA was extracted from 2 g of soil by using Qiagen's RNeasy Powersoil Total RNA Kit® according to the manufacturer's protocol. A DNase treatment using Qiagen's RNase-free DNase set was included after step 14 of the RNeasy Powersoil Total RNA Kit® protocol. The quality and quantity of the extracted RNA was assessed by Thermo Scientific™ NanoDrop 2000 (serial no. M125). After RNA extraction, reverse transcription was performed to synthesize cDNA using Qiagen's Omniscript RT Kit® and random nonamers from Integrated DNA Technologies. The cDNA was stored at -20 °C and used within one month. Backup cDNA aliquots were stored at -80 °C.

3.6.2 *Absolute quantitation of bacterial and archaeal genes relevant for N-cycling using reverse transcription and Droplet Digital PCR*

Quantitative PCR measurements were performed for a set of genes relevant to the nitrogen cycle for both bacteria and archaea allowing for the evaluation of the potential gene activity and expression in soil samples. Absolute quantitation of the expression of targeted genes was carried out by PCR amplification of cDNA using specific gene primers (Invitrogen) (**Table 3.4**) the Bio-Rad QX200™ Droplet Digital PCR™ (ddPCR™) system (serial no. 771BR1304) following Bio-Rad's QX200™ ddPCR™ Evagreen® Supermix protocol. The size of the amplicon for each gene primer set were verified via gel electrophoresis (**Figure 8.2**). Amplicons were sequenced using ABI sequencing to validate that the correct gene fragment was amplified. Optimized ddPCR™ reactions used 33ng/µL of cDNA. A typical 20-µL reaction used 10 µL of Evagreen Supermix, 2.5 µL

of 150 nM of target forward primer, 2.5 µL 150 nM of target reverse primer, 2.5 µL of RNase-DNase free water, and 2.5 µL of cDNA. A master mix containing all reaction components was made for each primer set. The master mix included the total number of samples (180) plus an additional 10% of the total for pipetting error. Once the reaction mixtures were prepared, 20 µL of each reaction was loaded into individual sample wells of a DG8™ Cartridge for the QX200™ Droplet Generator (Serial no. 772BR1305) followed by 70 µL of QX200™ Droplet Generator Oil for Evagreen® into the oil wells, according to the QX200 Droplet Generator Instruction Manual. After droplet generation, 40 µL of the droplets were carefully transferred into a 96-well PCR plate and sealed with a PX1 PCR Plate Sealer (Serial no. 770BR1714). The PCR thermocycling program recommended for the QX200™ ddPCR™ EvaGreen® Supermix was used: (1) enzyme activation at 95 °C for 5 mins, (2) 40 cycles of denaturation consisting of 30 sec at 95 °C, (3) 40 cycles of annealing/extension of 1 min of varying temperature according to the targeted primer set in **Table 3.4**, (4) a signal stabilization (i.e. ending the annealing temperature) of 4 °C for 5 min, and (5) then at 90 °C for 5 min to increase amplification specificity for 30 sec, with a ramp rate of 2 °C/sec. All quantitative PCR measurements were carried out in triplicate using the natural replication of the plot treatments. Three technical replicates for each depth and month were performed for the negative (no template) controls and resulted in no product amplification for all reactions described. After thermocycling, samples were placed into the QX200™ Droplet Reader (Serial no. 771BR1304) for absolute quantitation using the QuantaSoft™ software (V 1.4).

Table 3.4. *Gene sets used for quantitative PCR measurements relevant to the nitrogen cycle of Bacteria and Archaea.*

Gene name	Abbreviation	Primer ID	Primer sequence (5' to 3')	Annealing temperature (°C)	Reference
Nitrogenase reductase	*nifH*	PolF	TGCGAYCCSAARGCBGACTC	55	Poly et al., 2001
		PolR	ATSGCCATCATYTCRCCGGA		
Ammonia oxidizing bacteria	*amoB*	Bact-amoA-1F	GGGGTTTCTACTGGTGGT	47	Caffrey et al., 2007; Koops et al., 2006
		Bact-amoAr-R	CCCCTCBGSAAAVCCTTCTTC		
Ammonia oxidizing archaea	*amoA*	Arch-amoA-F	CTGAYTGGGCYTGGACATC	58-59	Coolen et al., 2006; Wuchter et al., 2006
		Arch-amoA-R	TTCTTCTTTGTTGCCCAGTA		
Proteobacterial membrane-bound nitrate reductase	*narG*	narG-F	TCGCCSATYCCGGCSATGTC	58	Bru et al., 2007
		narG-R	GAGTTGTACCAGTCRGCSGAYTCSG		
Proteobacterial periplasmic nitrate reductase	*napA*	V17m	TGGACVATGGGYTTYAAYC	61	
		napA4R	ACYTCRCGHGCVGTRCCRCA		
Denitrifying nitrite reductase; Cu-containing enzyme decoded	*nirK*	nirK876	ATYGGCGGVCAYGGCGA	59	Henry et al., 2004, 2006
		nirK1040	GCCTCGATCAGRTTRTGGTT		
Denitrifying nitrite reductase; cytochrome cd1 decoded	*nirS*	nirSCd3aF	AACGYSAAGGARACSGG	57	Throbäck et al., 2004
		nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA		
Nitrous oxide reductase 1	*nosZ*	nosZ1F	WCSYTGTTCMTCGACAGCCAGG	61	Henry et al., 2006
		nosZ1R	ATGTCGATCARCTGVKCRTTYTC		
Archaeal 16S rDNA	*Archaea*	ARCH1-1369F	CGGTGAATACGTCCCTGC	59	Suzuki et al., 2000
		PROK 1541R	AAGGAGGTGATCCRGCCGCA		
Bacteria 16S rDNA	*Bacteria*	BACT1369F	CGGTGAATACGTTCYCGG	56	
		PROK 1492R	CGWTACCTTGTTACGACTT		

3.7 STATISTICAL METHODS

3.7.1 *Differences in environmental and biotic parameters between land-use regimes at each depth and sampling date*

All statistical tests were performed on R version 3.5.2 (Eggshell Igloo). A 2-way analysis of variance (ANOVA) was performed to determine the effects of soil/crop management regimes, depth and their possible interaction on the soil bulk density and total porosity. A 3-way ANOVA was performed to determine the effects of sampling date (months), soil/crop management regimes, depth and their possible interaction on the environmental (soil pH, total carbon, NH_4^+-N, NO_3^--N, and water-filled pore space) and biotic (*nifH*, AOA- and AOB-*amoA*, *narG*, *napA*, *nirK*, *nirS*, and *nosZ1*) parameters. For the environmental parameters, the sampling dates, soil/crop management regimes, depth and their possible interaction, means were compared by performing post-hoc Tukey honestly significant difference (HSD) tests.

3.7.2 *Winter gene expression exploratory analysis*

It was hypothesised that increases in N-cycling gene profiles (*nifH*, AOA- and AOB-*amoA*, *narG*, *napA*, *nirK*, *nirS*, and *nosZ1)* exposed to cold conditions during the non-growing season are likely to occur in December, March and June, and could be related to conventional soil/crop management regimes typical of Newfoundland agriculture. The goal was not to look for differences in individual gene expression but to explore which genes were responsible for the pattern in the dataset. To this an LDA was performed to evaluate the discriminant structure of the dataset as driven by the gene expression profiles (i.e., grouping for December, March and June, and treatments within respective months). December represents the end of the fall with stability in gene expression, March represented winter with an increase in expression and June

represented spring with a decrease and stability in gene expression. An LDA is an exploratory analysis that employs a linearized combination of continuous variables, i.e. independent variables, to explain categorical dependent variable(s). Thus, it may be used with a series of independent variables to maximize the distances between classes or categories of responses. It is in a way related but a reverse of ANOVA which uses categorical independent variables (i.e., factors or categories) to describe continuous dependent variables. An LDA is interpreted in a similar manner as a logistic or principal component analysis, where samples are classified/grouped in categories with similar relationship to the linear discriminant vectors, and the apparent degree of collinearity among the explanatory variables can be directly visualised on linear discriminant vectors (akin to principal component eigenvectors). As such to determine the most influential driving variables that best explains the separation among categories, the N-cycling gene profiles during the respective sampling dates and treatments within each month were added to the LDA biplots as vector arrows. However, due to the low number of replications within each month the analysis was not separated by depth and instead focused on the overall dataset within sampling dates. Values of the N-cycling gene profiles were Log_{10} normalised prior to the analysis.

3.7.3 *Relationships between environmental and N-cycling gene profiles*

Following the 3-way ANOVA, a Canonical Correspondence Analysis (CCA) was performed to determine if the changes in transcript abundances of all genes during the non-growing season can be coupled to the categorical factors (sampling time or soil/crop management regimes). To reduce the analytical load of depth as a confounding factor in the CCA, the tests were separated by depth 1 and 2. The dynamic changes in soil parameters (soil

pH, total carbon, NH_4^+-N, NO_3^--N, and water-filled pore space) in association with the categorical factors during the non-growing season were added to the CCA biplots as vector arrows to determine the most influential driving variables and their relative contributions to changes in gene transcript abundance. Thereafter, by averaging across all sampling dates and soil/crop management regimes, correlation matrices were constructed for each depth to determine the strength of the associations, by using Pearson's Rho (r), between the changes in gene transcript abundance profiles with changes in abiotic and biotic parameters. Once the matrices were assessed for the most influential variables, a handful of simple linear models and their resulting scatter plots were constructed following the assessment of the most influential variables to visualize the biological significance (r^2) of the models.

4 RESULTS

4.1 CLIMATIC CONDITIONS DURING WINTER

Hobo ® data loggers recorded the daily air and soil temperatures for each sampling event. In St. John's, Newfoundland the field experimental plots were sporadically covered in snow from mid-December, 2017 to the end of March, 2018 with a decrease in average daily air temperature to below 0 °C and average daily soil temperatures to around 0 °C at the plough layer (12 cm, **Figure 4.1** and **Table 4.7**). Though data is sparse, the snow cover period was from December 17th, 2017 to April 15th, 2018 with the average daily snow depth peaking at 23 cm on February 27th, 2018 (https://bit.ly/2UV0Xv6; **Figure 4.1** and **Table 4.1**). Snow cover was present on the February (1 cm) and March (7 cm; https://bit.ly/2UV0Xv6; **Figure 4.1** and **Table 4.1**) sampling events only. A consistently deep snowpack was present for the N-V forest plots (data not shown). The average daily air temperature for the day of sampling decreased from 13.2 °C in October 2017 to 1.5 °C in December, decreased further from 1.5 °C in December to -1.5 °C in February, stabilised between February (-1.5 °C) and March (-1.8 °C) 2018; gradually increased from -1.8 °C in March to 3.5 °C in May 2018; and stabilised above 0 °C in June 2018 (6.0 °C, **Figure 4.1** and **Table 4.1**). The 2018 winter and spring seasons measured average daily soil temperatures at 12 cm depth remained close to 0 °C during the snow cover period (**Figure 4.1** and **Table 4.1**). However, there was a sharp rise in average daily soil temperature from March to May; shortly after which the average daily soil temperature was eventually raised above 0 °C between May and June (**Figure 4.1** and **Table 4.1**). The snowmelt period was from mid-March to mid-April of 2018 (**Figure 4.1**). The soils experienced repeated FTCs at the beginning of the snow cover period and during the snowmelt period, where the soil and air temperatures were above 0 °C during the day and near/below 0 °C during the night (mid-

December to mid-April). Intense periods of FTCs occurred: (1) in the winter from late-January to mid-February, and (2) in the spring from mid-March to mid-April, which coincided with the snowmelt period. The lowest average daily air temperature recorded during the non-growing season was on February 23rd (-9.0 °C) while the lowest soil temperature was recorded on February 26th, 2018 (-0.8 °C; data not shown).

Table 4.1. *Average daily air and soil plough layer (depth 1, 12 cm) temperature (°C) for the day of sampling in October, December, February, March, May and June.*

Day/Month	AVRG Daily Air T (°C)	AVRG Daily Soil T (°C)	AVRG daily Snow cover (cm)
5-Oct-17	13.15 ± 0.35		0
12-Dec-17	1.47 ± 0.32		0
7-Feb-18	-1.54 ± 0.35	0.17 ± 0.00	1
9-Mar-18	-1.75 ± 0.21	0.00 ± 0.00	7
10-May-18	3.54 ± 0.68	7.20 ± 0.02	0
5-Jun-18	5.98 ± 0.77	6.59 ± 0.02	0

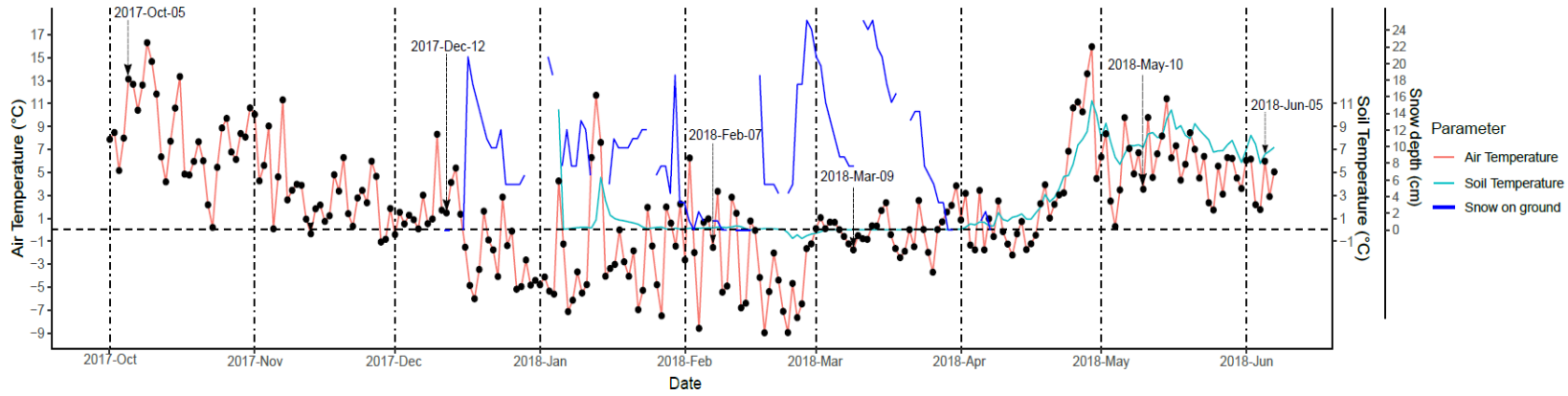


Figure 4.1. *Line graph of the average daily air (red line) and soil plough layer (green line; depth 1, 12 cm) temperature (°C), and snow depth (blue line) for the day of sampling (indicated by arrows) in October, December, February, March, May and June.*

4.2 BASIC SOIL PROPERTIES DURING WINTER

4.2.1 *Soil bulk density and total porosity*

Since the experimental plots were under a no till farming practice, it was expected that the soil bulk density and total porosity (St) would remain practically stable between treatment and depths in October of 2017. However, significant differences among the treatment × depth interaction were observed for bulk density (*p*-value = <0.01, **Table 4.3**). At depth 1, the soil bulk density of N-V was significantly different from the other treatments, with an average of 0.38 g/cm^3 compared to the average of 0.91, 1.05, 0.80 and 0.92 g/cm^3 respectively. At depth 2, the soil bulk density of C-G was significantly different from C-F only, with an average of 1.25 compared to an average of 0.82 g/cm^3 respectively (**Figure 4.2 A**). There were significant differences in soil bulk densities between depths for N-V only, with an average of 0.38 g/cm^3 compared to an average of 1.00 g/cm^3 (**Figure 4.2 B**).

Table 4.2. *Average soil physical properties (porosity [% vol] and bulk density [g/cm^3]) among treatments described in* ***Table 3.1****. at depth 1 (0-25 cm) and 2 (25-40 cm). Values are mean ± standard error.*

Treatment and plots	Bulk density		Porosity (St; %)	
	Depth 1	Depth 2	Depth 1	Depth 2
N-V (Native Vegetation) F1-3	0.38 ± 0.00	1.00 ± 0.00	73.13 ± 0.00	60.09 ± 0.00
C-G (Conventional Grass) 2, 5 and 9	0.91 ± 0.04	1.25 ± 0.54	63.62 ± 1.49	51.07 ± 21.15
I-G (Improved Grass) 1, 4 and 8	1.05 ± 0.08	1.06 ± 0.12	58.26 ± 3.33	58.22 ± 4.63
C-F (Conventional Forage) 6, 10 and 11	0.80 ± 0.14	0.82 ± 0.17	68.20 ± 5.59	67.91 ± 6.55
I-F (Improved Forage) 3, 7 and 12	0.90 ± 0.18	1.07 ± 0.16	64.14 ± 7.22	58.13 ± 6.38
Total	0.81 ± 0.09	1.04 ± 0.20	65.47 ± 3.53	59.08 ± 7.74

Table 4.3. *Two-way ANOVA output to test the significances of depths and treatments and their interaction term for the differences in average soil bulk density (g/cm^3) and total porosity (% vol).*

Factor	Bulk density	Total porosity
Depth	<0.01	<0.01
Trt	<0.01	<0.01
Depth*Trt	<0.01	0.07
n	171	171

On the other hand, there were significant differences among the treatment and soil depths in total porosity respectively (*p*-value = <0.01 each, **Table 4.3**), but no interaction effect. Total porosity at depth 1 is significantly different from depth 2, with an average of 65.1 % compared to an average of 58.9% (**Figure 4.3 A**). The soil total porosity of C-G is significantly different from C-F only across both depths, with an average of 57.4% compared to an average of 68.1% respectively (**Figure 4.3 B**).

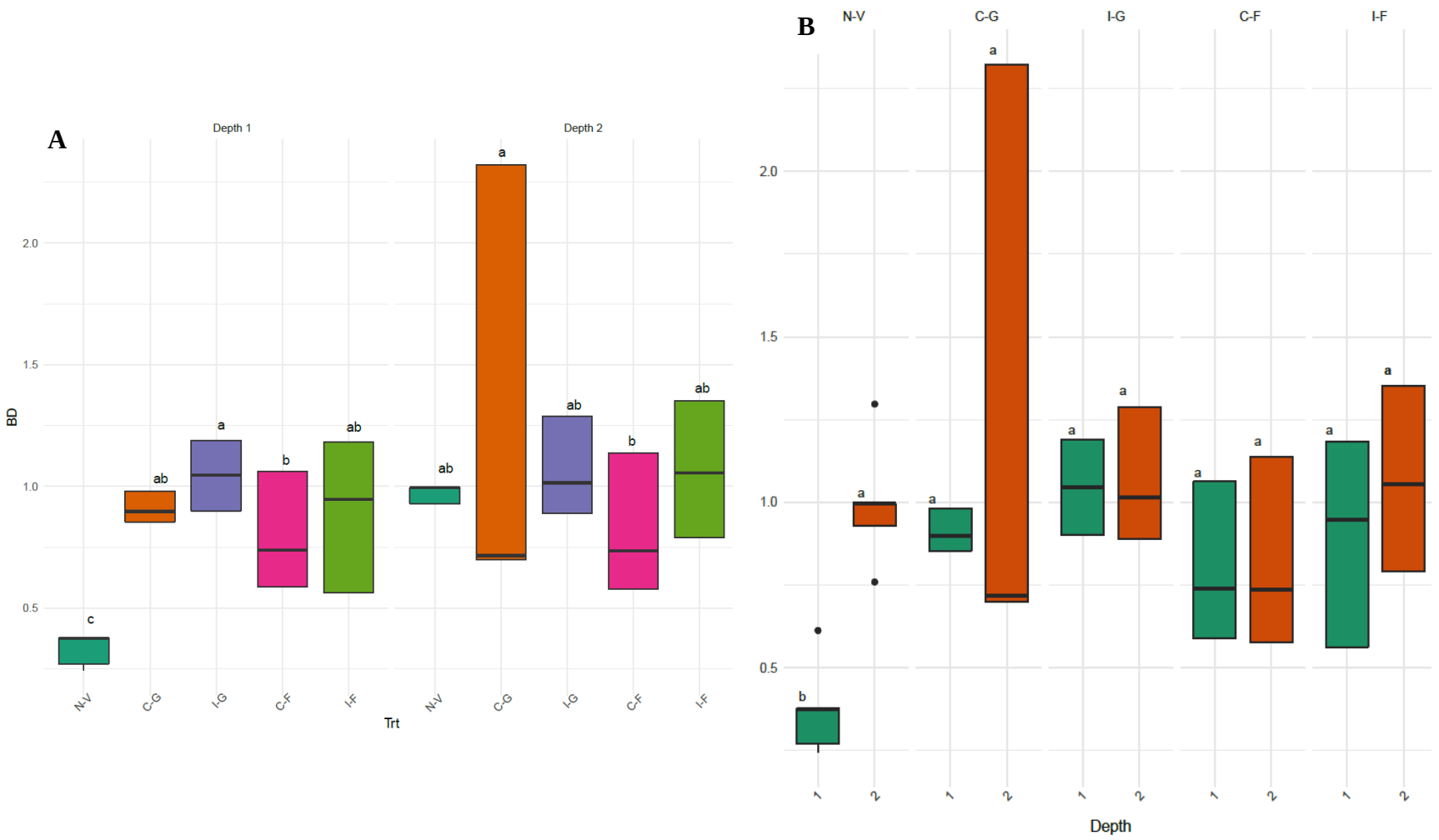


Figure 4.2. *Boxplots summarizing the significant differences in mean soil bulk densities (BD) among the treatments described in* ***Table 3.1.*** *at depth 1 (0-25 cm) and 2 (25-40 cm). In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Significant differences for the depth × treatment interaction are represented in two ways: (A) differences in mean among depth 1 and 2 across all treatments; and (B) differences in mean among depths for each treatment. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$).*

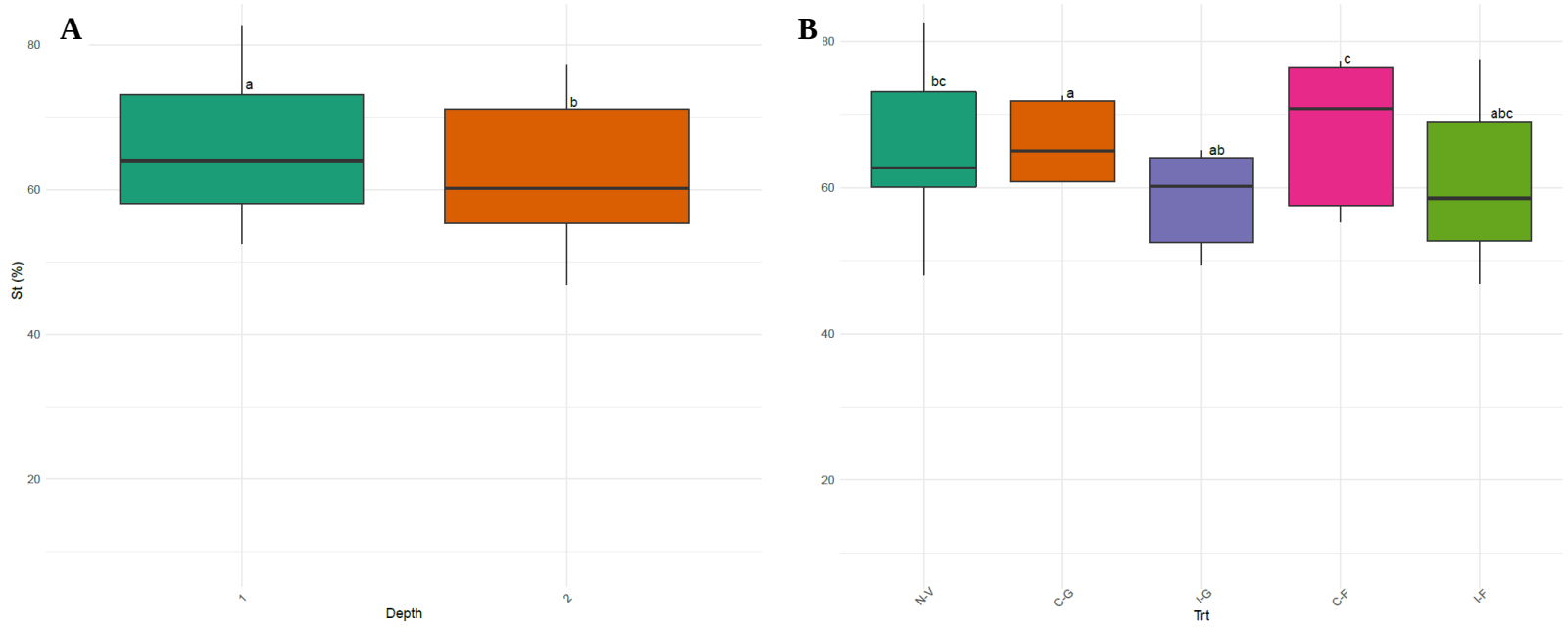


Figure 4.3. *Boxplots summarizing the significant differences in mean soil total porosities (St) at depth 1 (0-25 cm) and 2 (25-40 cm) and the treatments described in* ***Table 3.1****. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Significant differences are represented in two ways: (A) differences in mean among depth 1 and 2 across all treatments; and (B) differences in mean among treatments across both depths. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$).*

4.2.2 *Winter dynamics of soil WFPS, pH, NH_4^+, NO_3^- and total carbon supply*

For all treatments and both depths, soil WFPS, NH_4^+ NO_3^- and TC concentrations and pH changed over time. Dynamic changes in the environmental variables were, in most part, significantly related to time (**Table 4.4**). The most notable temporal changes was an overall decrease in NH_4^+, NO_3^- and TC concentrations, and pH at both depths in all treatments from October to December of 2017. Soil WFPS at both depths and TC for the N-V treatment at depth 1 increased from October to December of 2017 (**Figure 4.4 A-E**). Then there was an overall increases in soil WFPS, NH_4^+ and NO_3^- concentrations, and pH which occurred in mid-winter (February and March) and June 2018; whereas soil TC concentrations decreased further from February to March, with a subsequent increase in June of 2018 (**Figure 4.4 A-E**).

Table 4.4. *Three-way ANOVA output to test the significances of the depths, sampling dates, treatments and their interaction term for the differences in average soil water-filled pore space (WFPS; %), pH NH_4^+-N and NO_3^--N, and total carbon concentrations (TC; µg/mg Element g^{-1} soil/g^{-1} air dry soil) over the sampling period under changing environmental conditions at depth 1 (0-25 cm) and 2 (25-40 cm).*

Factor	WFPS	pH	NH_4^+	NO_3^-	TC
Depth	0.18	0.77	0.03	0.07	<0.01
Month	<0.01	<0.01	<0.01	<0.01	<0.01
Trt	<0.01	<0.01	<0.01	<0.01	<0.01
Depth*Month	0.61	0.03	0.26	0.54	0.91
Depth*Trt	<0.01	<0.01	0.04	0.18	<0.01
Month*Trt	1.00	<0.01	0.03	<0.01	0.93
Depth*Month*Trt	1.00	0.12	0.74	0.96	1.00
n	171	171	171	171	171

Figure 4.4. *Box and line plots summarizing dynamic changes in soil (A) WFPS, (B) NH_4^+, (C) $NO_3^-$and (D)TC concentrations, and (E) pH for treatments described in* ***Table 3.1*** *for the individual sampling dates at depth 1 (0-25 cm) and 2 (25-40 cm). In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. The lines and points passing through the boxplots are monthly mean (n=3) values for each treatment.*

4.2.2.1 Soil WFPS

A significant depth × treatment interaction and time effect on soil WFPS was found (*p*-value = <0.01 each, **Table 4.4**). At depth 1, the soil WFPS in the I-G treatment is significantly different from the N-V and C-F treatments only, with an average of 89.3% compared to an average of 53.3 and 65.0 % respectively. At depth 2, the soil WFPS in N-V is significantly different from C-F only with an average of 91.8% compared to an average of 64.7% at depth 2 (**Figure 4.5 A**). There significant differences in soil WFPS between depths for the N-V treatment only, with an mean of 53.3% for depth 1 compared to a mean of 91.8 % for depth 2 (Figure 4.5 B). March is significantly different from October, December, February, May and June (mean of 95.2% compared to a mean of 67.4, 71.0, 74.7, 69.8 and 74.4% respectively, **Figure 4.6**). Soil WFPS slowly increased from October-March (67.4 to 95.2%), decreased from March to May (95.2 to 69.8 %) and then increased once more from May to June (69.8 to 74.4 %; **Figure 4.6**).

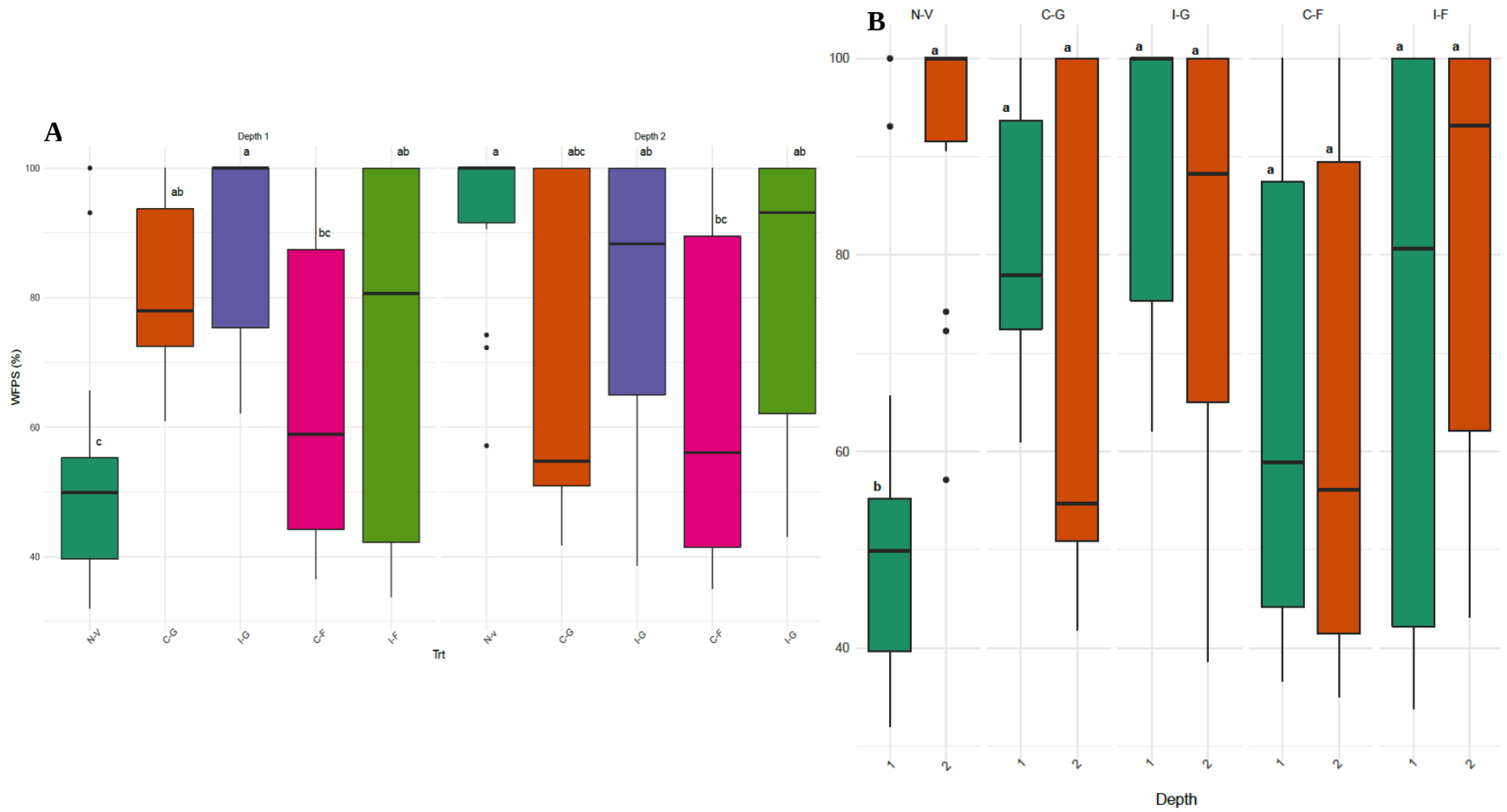


Figure 4.5. *Boxplots summarizing significant differences in mean soil water-filled-pore-space (WFPS among treatments described in* ***Table 3.1****. at depth 1 (0-25 cm) and 2 (25-40 cm) across all sampling dates. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Significant differences for the treatment × depth interaction across all sampling dates are represented in two ways: (A) differences in mean among the treatments at depth 1 and 2 ; and (B) differences in mean among depths for each treatment. Boxplots with the same letter code are not significantly different (Tukey HSD, p < 0.05).*

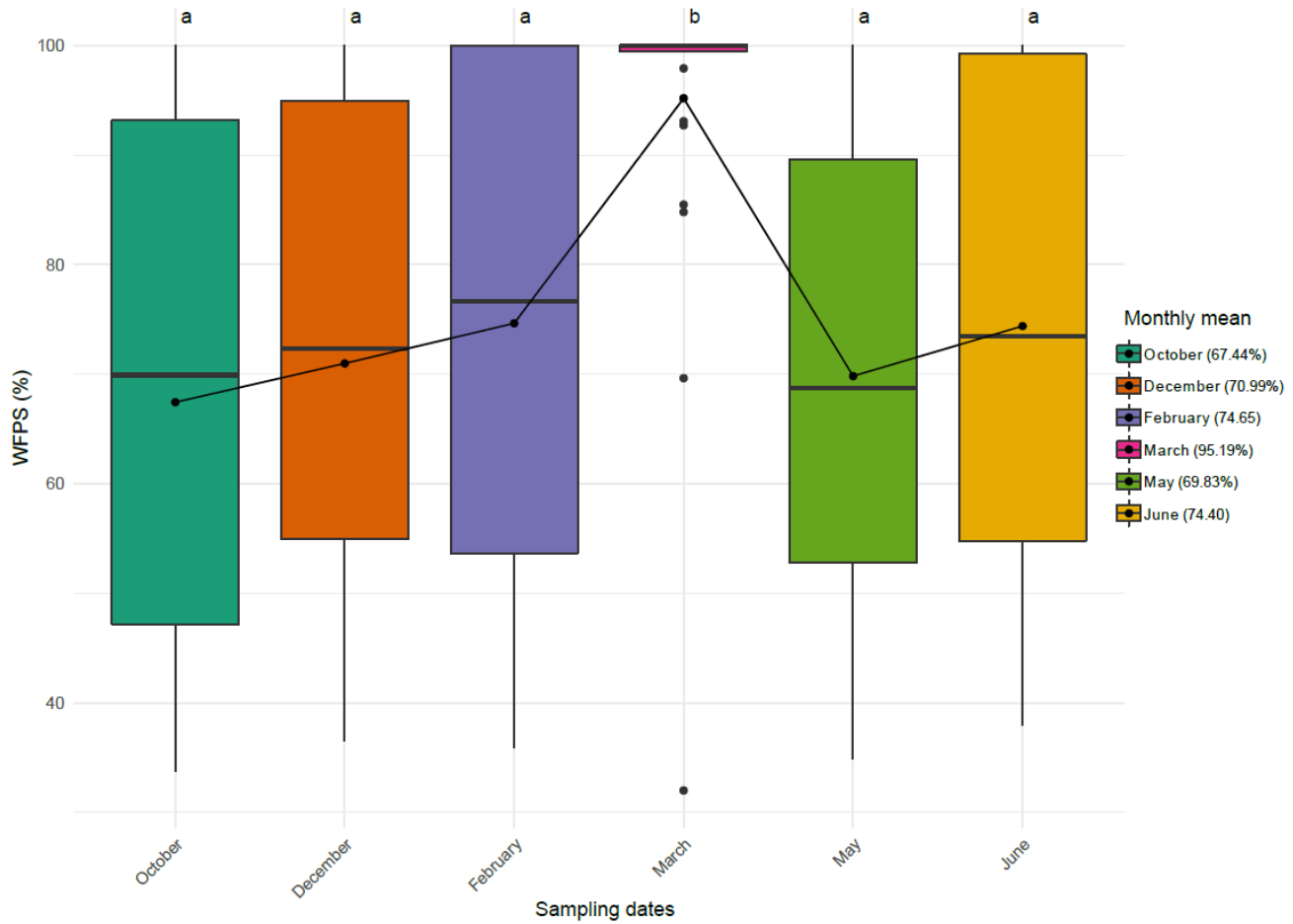


Figure 4.6. *Boxplots summarizing significant differences in mean soil water-filled-pore-space (WFPS) among sampling dates. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. The legend in the figure shows the monthly mean WFPS (n=30) values. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$).*

4.2.2.2 Soil pH

Significant depth × month, depth × treatment, and month × treatment interaction effects were found on soil pH (*p*-value = 0.03, <0.01 and <0.01 respectively, **Table 4.4**). Though we found a significant depth × month interaction effect, due to the variability in the dataset the post hoc Tukey HSD was not robust enough to capture the differences in mean between months at each depth (**Figure 4.7 A**).; however, there were significant differences between depth 1 and 2 for the October, December and February sampling dates only (mean of 5.99 vs. 6.12, 5.71 vs. 5.63, and 5.69 vs. 5.44 respectively, **Figure 4.7 B**). N-V is significantly different from the other treatments at both depths; with a mean of 3.77 compared to a treatment mean of 6.09 at depth 1, and a mean of 4.27 compared to a treatment mean of 5.98 (**Figure 4.8 A**). Significant differences in soil pH between depths were found for the N-V treatment only, with a mean of 3.77 for depth 1 compared to a mean of 4.27 for depth 2 (**Figure 4.8 B**). The soil pH for the N-V treatment is significantly different from the other treatments throughout the sampling dates when averaged over both depths (**Figure 4.9 A**). There were significant differences in soil pH between: March and the other sampling dates for C-G and I-F; May and June with March only for I-G; October, May and June with December, February and March for C-F; and March (**Figure 4.9 B**). When averaged across both depths, soil pH in treatments C-G, I-G, C-F and I-G slowly decreased from October to February, increased from February to March, then decreased from March to May but stabilised from May to June; whereas in N-V the soil pH decreased from October to December, increased from December to February and then remained stable for the rest of the season from February to June of 2018 (**Figure 4.9 A**).

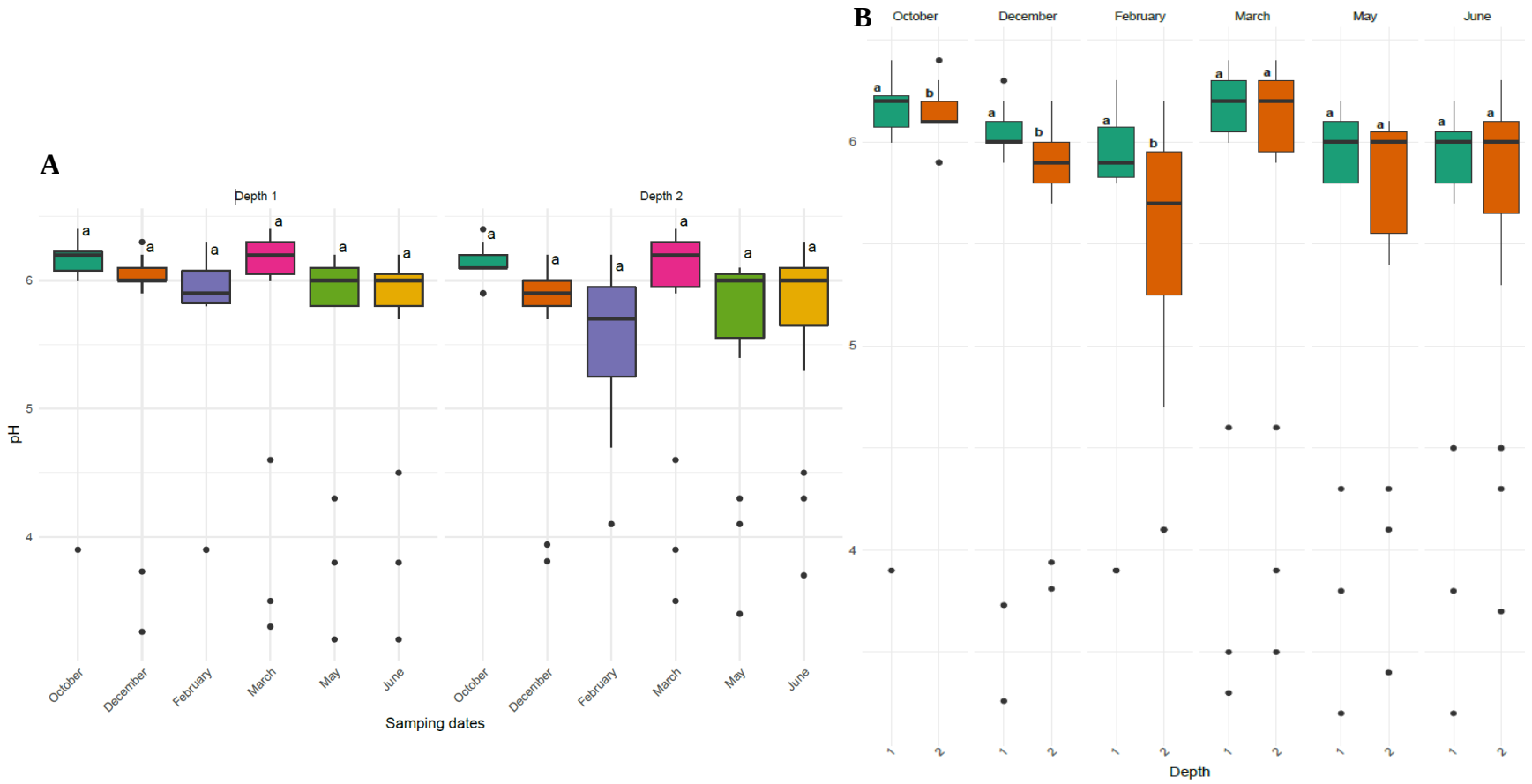


Figure 4.7. *Boxplots summarizing the significant differences in mean soil pH among sampling dates at depth 1 (0-25 cm) and 2 (25-40 cm). In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Lines and points passing through the boxplots are monthly mean (n=6) values for each treatment. Significant differences for the depth × month interaction across all treatments are represented in two ways: (A) differences in mean among sampling dates at each depth; (B) differences in mean among depth at each sampling date. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$).*

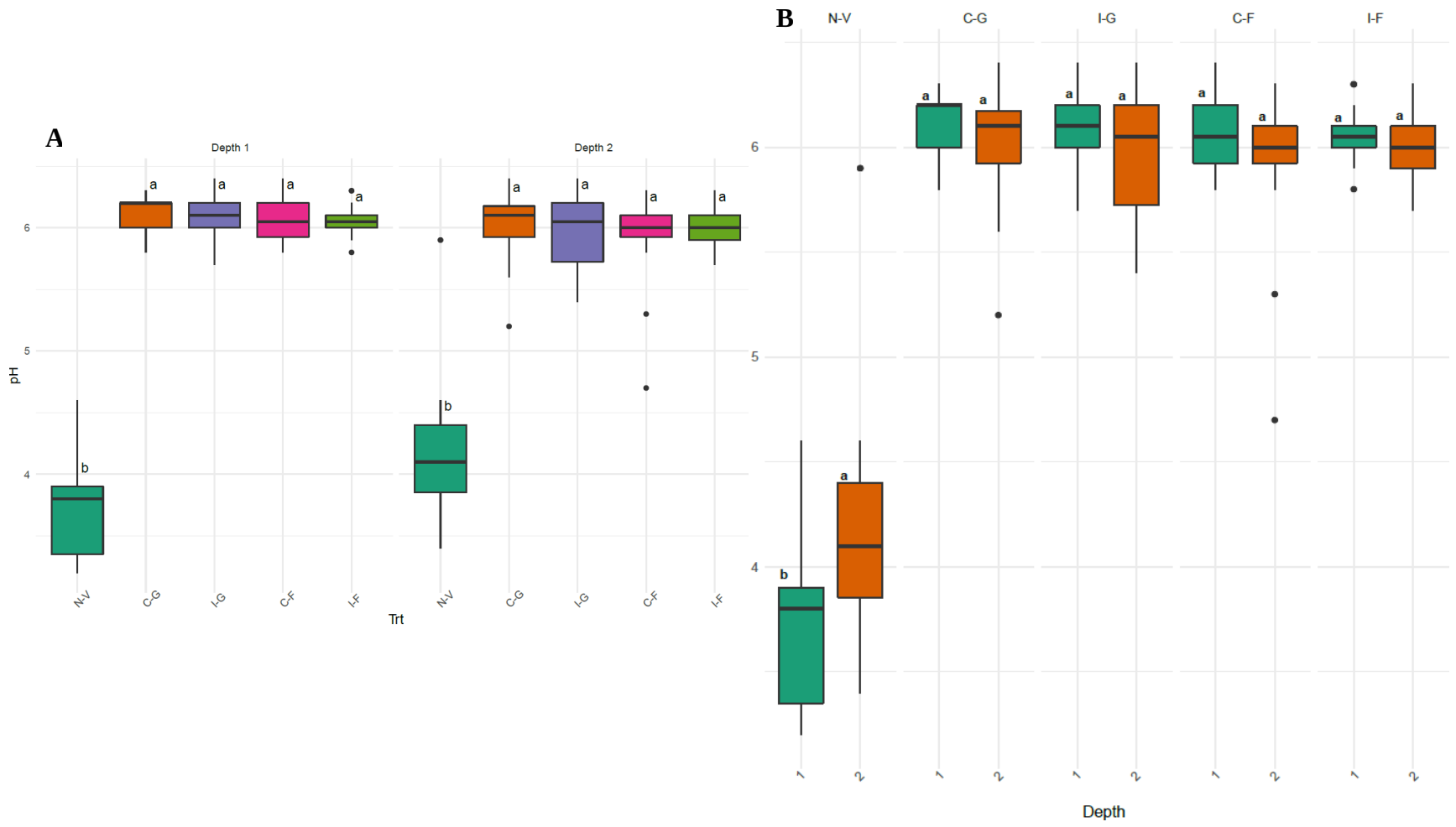
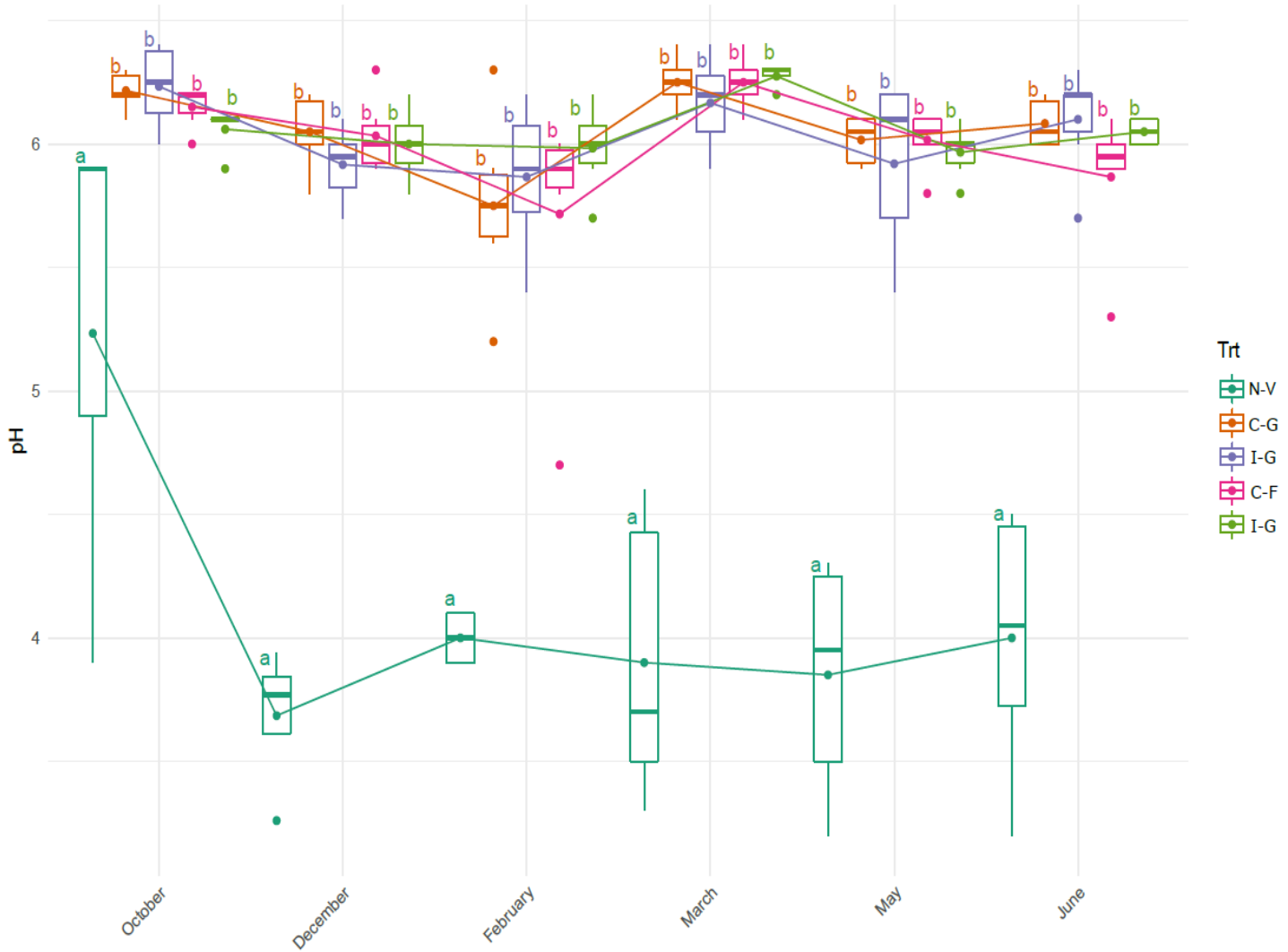


Figure 4.8. *Boxplots summarizing the significant differences in soil pH among treatments described in* ***Table 3.1*** *at depth 1 (0-25 cm) and 2 (25-40 cm). In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Significant differences for the depth × treatment interactions across all sampling dates are represented in two ways: (A) differences in mean among the treatments at each depth; (B) differences in mean among depth for each treatment. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$).*

A


pH
6
5
4
October
December
February
March
May
June
Trt
N-V
C-G
I-G
C-F
I-G

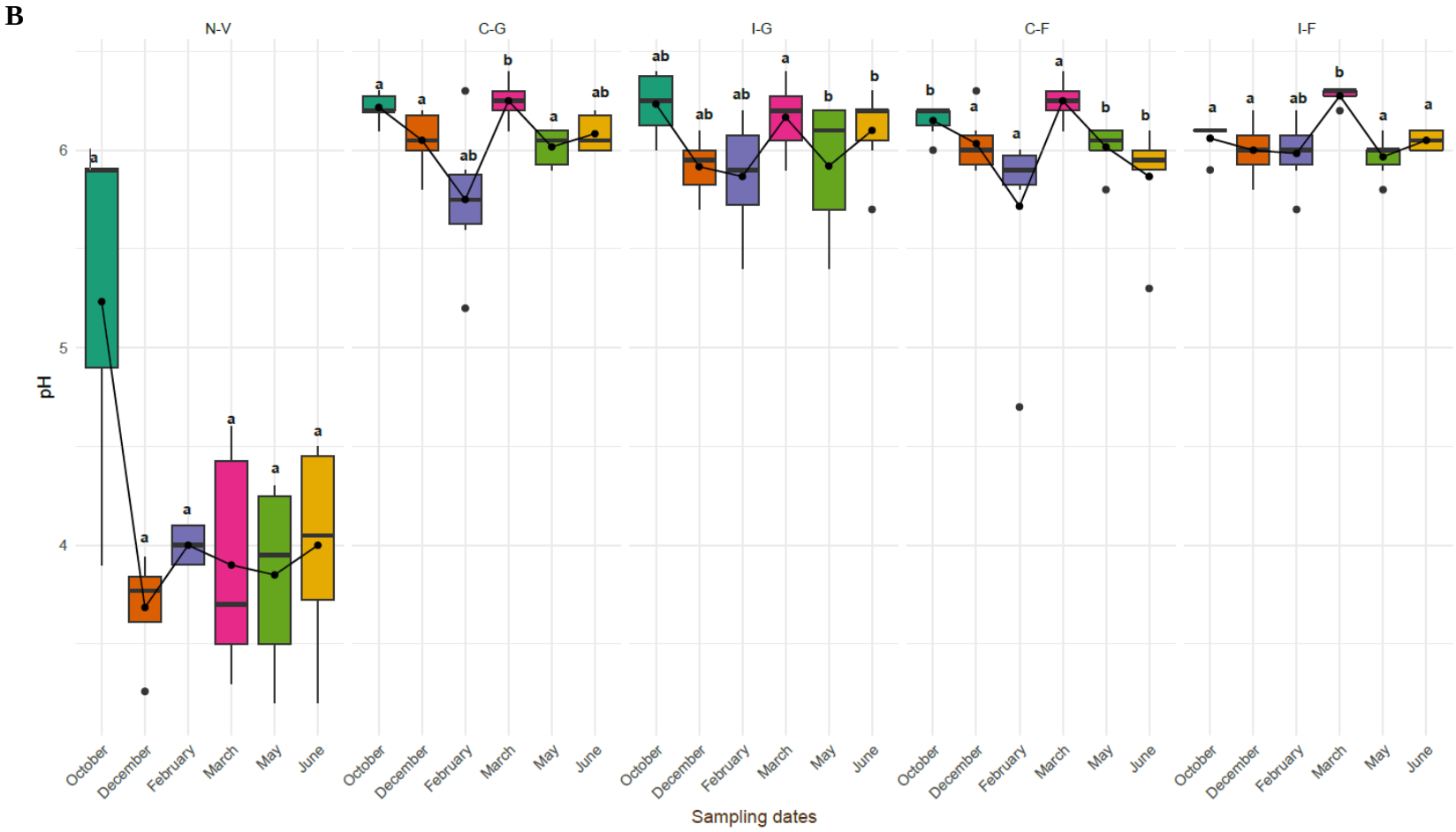


Figure 4.9. *Boxplots summarizing the significant differences in soil pH among treatments described in* ***Table 3.1*** *and sampling dates. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Lines and points passing through the boxplots are monthly mean (n=6) values for each treatment. Significant differences for the month × treatment interaction across both depths are represented in two ways: (A) differences in mean among treatments at each sampling date; (B) differences in mean among sampling dates for each treatment. Boxplots with the same letter code are not significantly different (Tukey HSD, p < 0.05).*

4.2.2.3 Soil inorganic nitrogen (NH_4^+- and NO_3^--N)

Significant depth × treatment and time × treatment interaction effects were found for soil NH_4^+-N concentrations (*p*-value = 0.03 and 0.04 respectively, **Table 4.4**). At depth 2 only, N-V is significantly different from the other treatments, with an average of 25.83 µg N g soil $^{-1}$ compared to an average of 13.85, 12.98, 13.92, and 11.71 µg N g soil $^{-1}$ respectively (**Figure 4.10 A**). There were significant differences between depths for the I-G treatment only, with a mean of 20.93 µg N g soil $^{-1}$ for depth 1 compared to an average of 12.98 µg N g soil $^{-1}$ for depth 2 (**Figure 4.10 B**). The soil NH_4^+-N concentrations in the N-V treatment was significantly different from the C-G and I-F treatments in May only, with an average of 22.85 µg N g soil $^{-1}$ compared to a treatment average of 15.46 µg N g soil $^{-1}$ (**Figure 4.11 A**). There were significant differences in soil NH_4^+-N concentrations between: March vs. December, May vs. June sampling dates for N-V; February vs. December and vs. May for C-G; February vs. May only for I-G, March with the other sampling dates except February for C-F; and May vs. February and vs. March only for I-F (**Figure 4.11 B**). When averaged across both depths and all treatments, soil NH_4^+-N concentrations fluctuated throughout the sampling periods but when averaged across all treatments were lowest in December of 2017 and May of 2018 (mean of 11.04 and 7.96 µg N g soil $^{-1}$ respectively; whereas soil NH_4^+-N concentrations reached their peak values in February and March of 2018 (mean of 24.53 and 27.91 µg N g soil $^{-1}$ respectively; **Figure 4.11 A**).

A significant time × treatment interaction effect was found for soil NO_3^--N concentrations when averaged across both depths (*p*-value = <0.01, **Table 4.4**). Soil NO_3^- concentrations were significantly different between: N-V vs. C-G in October; N-V vs. C-G and I-G in December; N-V vs. C-G and vs. C-F vs. I-F in March; N-V vs. I-G, vs. C-F and vs. I-F in May only (**Figure 4.12 A**). Soil NO_3^- concentrations were significantly different between: December vs. June with

the other sampling dates for C-G; June vs. the other sampling dates for I-G and C-F; and February and June vs. the other sampling dates respectively for I-F (**Figure 4.12 B**). When averaged across both depths and all treatments, soil NO_3^- concentrations remained low for October (mean of 5.69 µg N g soil $^{-1}$), December (mean of 7.28 µg N g soil $^{-1}$), March (mean of 3.68 µg N g soil $^{-1}$) and May (mean of 6.39 µg N g soil $^{-1}$), but peaked in February and June (mean of 16.01 and 42.57 µg N g soil $^{-1}$ respectively; **Figure 4.12 A**).

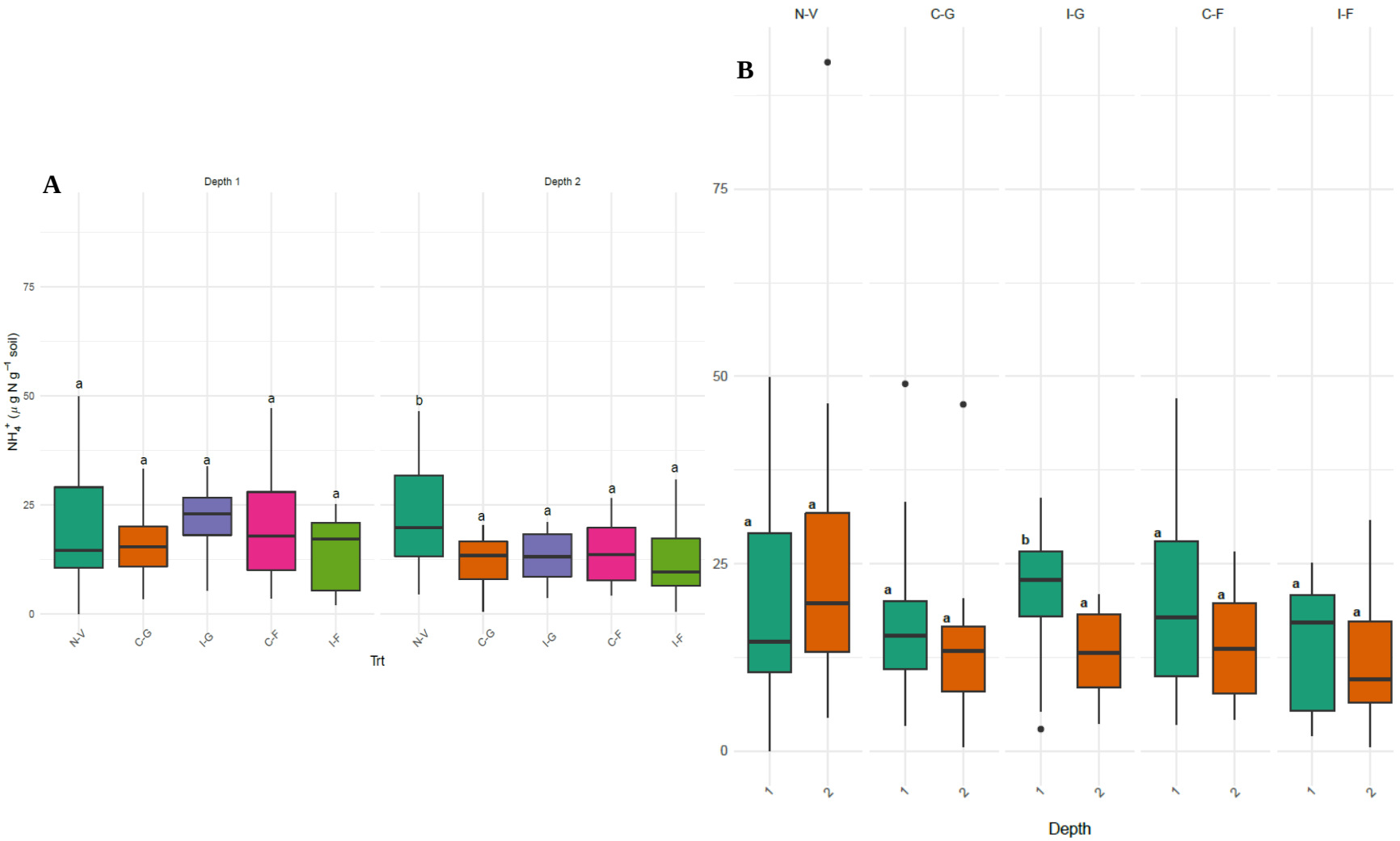
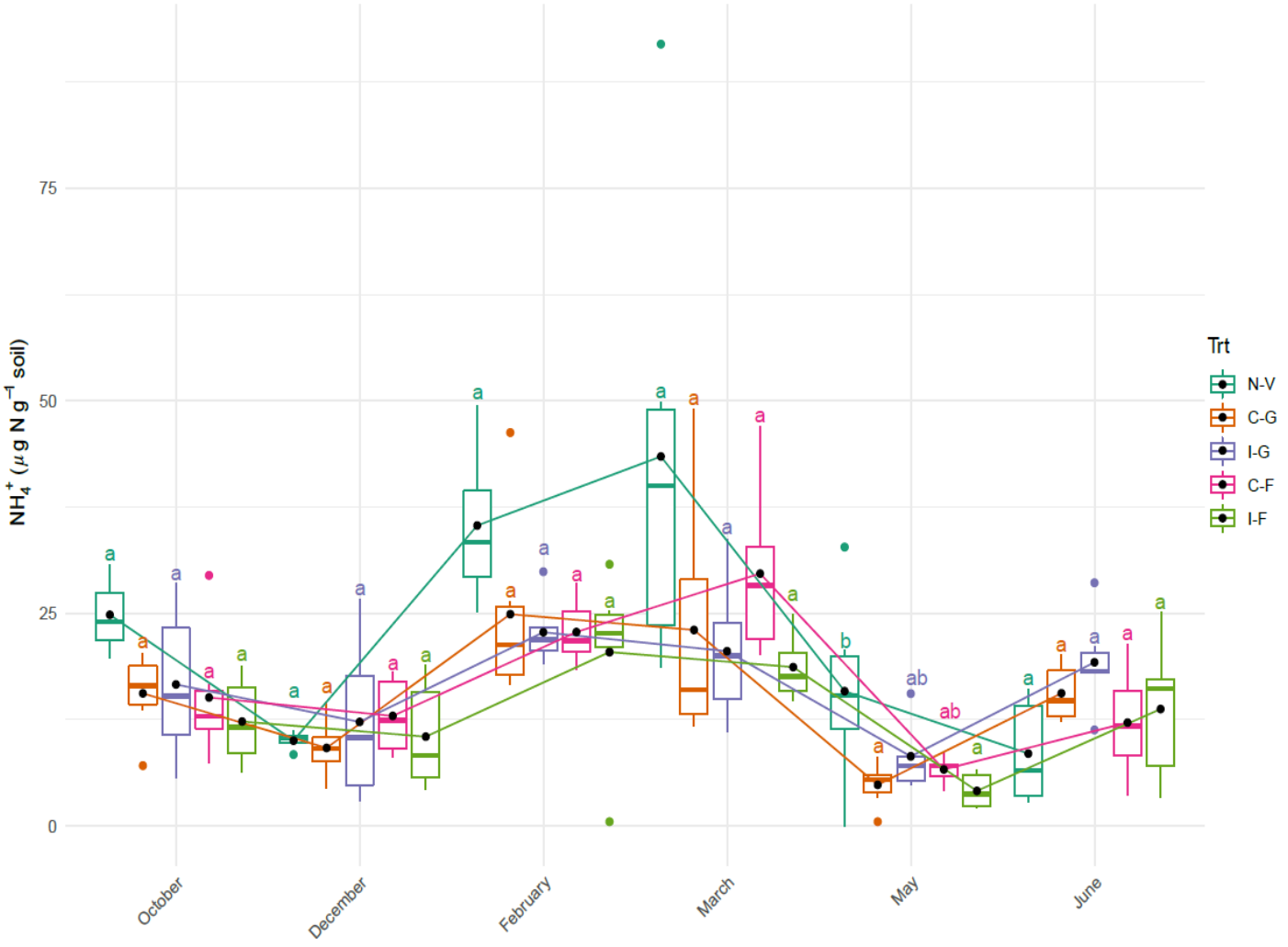


Figure 4.10. *Boxplots summarizing the significant differences in the soil NH_4^+ concentrations (µg N g^{-1} soil) among treatments described in* ***Table 3.1*** *at depth 1 (0-25 cm) and 2 (25-40 cm). In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Significant differences for the depth × treatment interaction across all sampling dates are represented in two ways: (A) differences in mean among treatments at each depth; (B) differences in mean among depths for each treatment. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$.*

A


75
50
25
0
NH_4^+ (μg N g^{-1} soil)
October
December
February
March
May
June
Trt
N-V
C-G
I-G
C-F
I-F

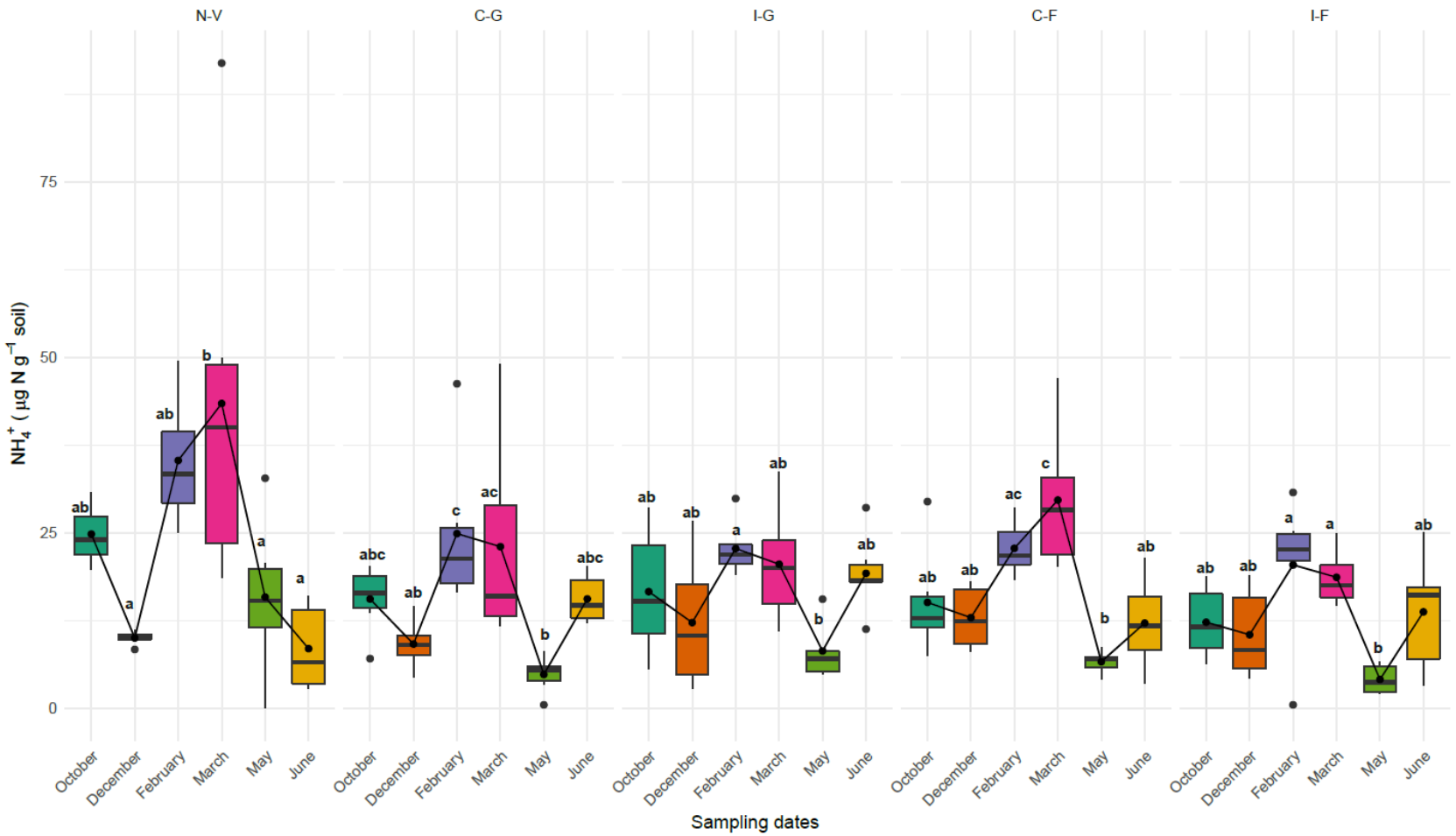
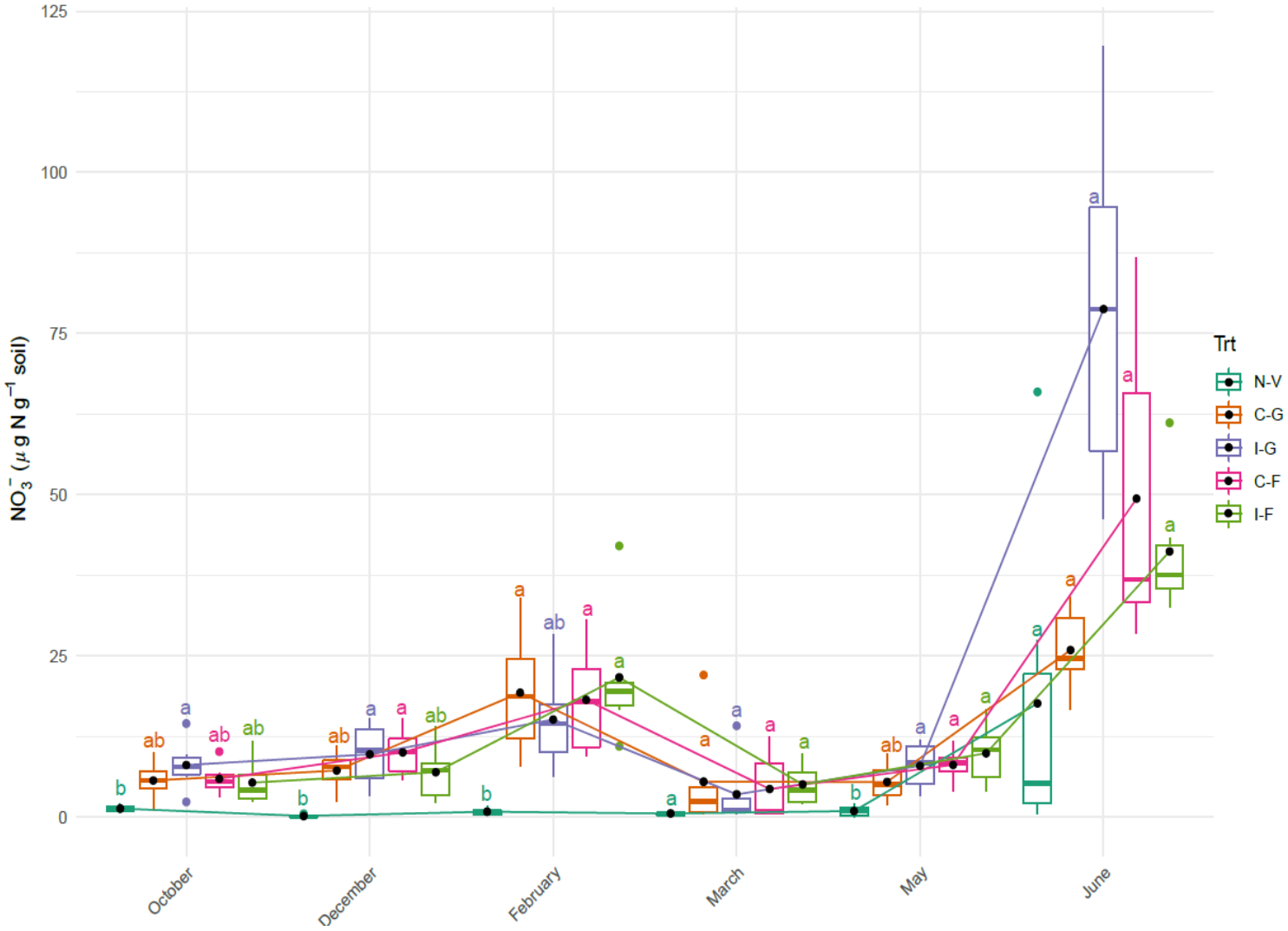


Figure 4.11. *Boxplots summarizing the significant differences in the soil NH_4^+ concentrations (µg N g^{-1} soil) among treatments described in* ***Table 3.1*** *at each sampling date. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Lines and points passing through the boxplots are monthly mean (n=6) values for each treatment. Significant differences for the month × treatment interaction across both depths are represented in two ways: (A) differences in mean among treatments for each sampling date; (B) differences in mean among sampling dates for each treatment. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$.*

A


125
100
75
50
25
0
NO_3^- (μg N g^{-1} soil)
October
December
February
March
May
June
Trt
N-V
C-G
I-G
C-F
I-F

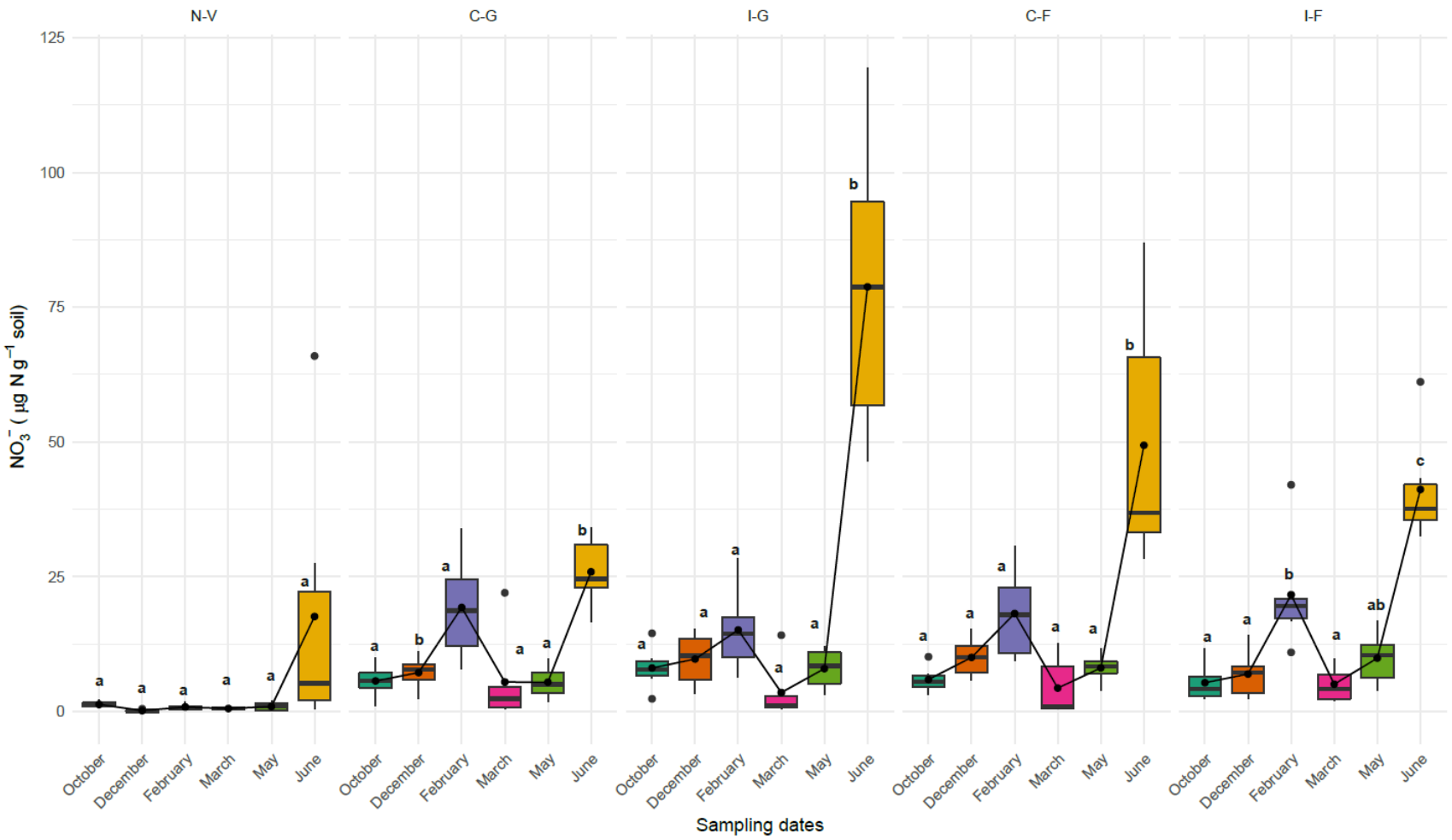


Figure 4.12. *Boxplots summarizing the significant differences in the soil NO_3^- concentrations (µg N g^{-1} soil) among treatments described in* ***Table 3.1*** *and sampling dates. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Lines and points passing through the boxplots are monthly mean (n=6) values for each treatment. Significant differences for the month × interaction across both depths are represented in two ways: (A) differences in mean among treatments for each sampling dates; (B) differences in mean among sampling dates for each treatment. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$.*

4.2.2.4 Soil TC and TN supply

Total nitrogen from the soil samples were below the detectable limit, i.e. negative values were obtained from the CHNS/O elemental analyzer. A negative value means that the samples were below the minimum reflective index in the frontal chromatograph (about 200 micrograms N mg^{-1} of soil) as it passes through the thermal conductivity detector of the CHNS/O. The inability to quantify the soil's total nitrogen was further confirmed since the inorganic nitrogen levels were about 0.10 mg /g of dry soil at their maximum. Although this does not include the organic N, the results suggest that the nitrogen in a 5 mg sample of dry soil may be one order of magnitude lower, below the detection limit of the CHNS/O elemental analyser.

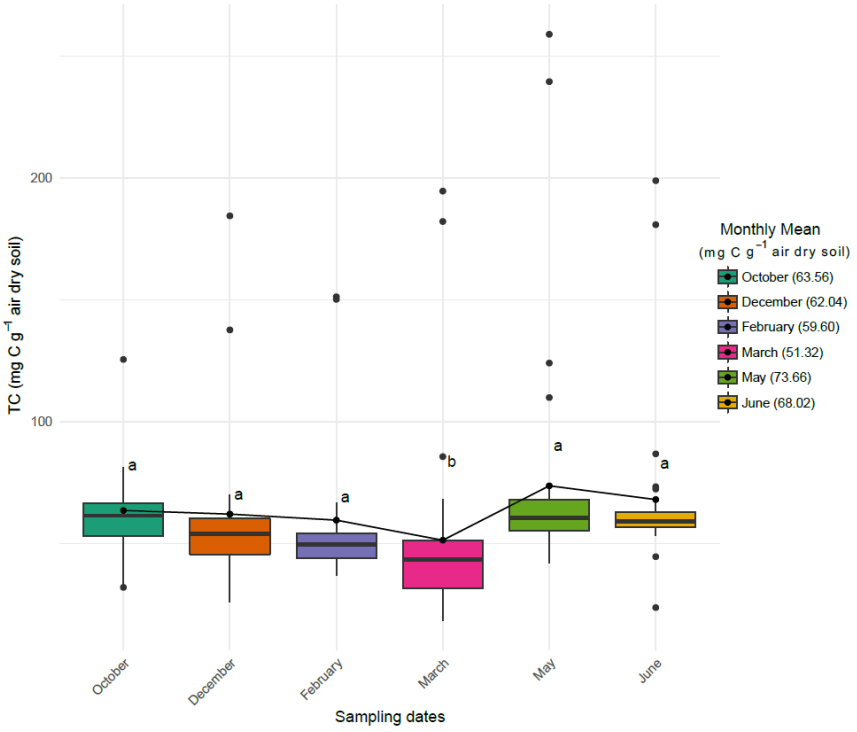


Figure 4.13. *Boxplots summarizing the significant differences in soil total carbon concentrations (TC; mg C g^{-1} air dry soil) among sampling dates across all depths and treatments described in* ***Table 3.1****. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. The legend in* the *figure shows* the *monthly mean TC (n=30) values. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$).*

Soil TC varied during the sampling year among the treatments. Nevertheless, there was a significant time and depth × treatment interaction effect on soil TC concentrations (*p*-value = <0.01 respectively, **Table 4.4**). When averaged across all treatments and depths, March TC is significantly lower than for the other months, with an average of 52.91 mg C g^{-1} air dry soil compared to an average of 61.49 (October), 58.82 (December), 56.39 (February), 76.12 (May) and 68.07 mg C g^{-1} air dry soil (June; **Figure 4.13**) respectively. Soil TC concentrations slowly decreased from October to March (61.49 to 52.91 mg C g^{-1} air dry soil), increased from March to May (52.91 to 76.12 mg C g^{-1} air dry soil), and decreased once more from May to June (76.12 to 68. 06 mg C g^{-1} air dry soil; **Figure 4.13**). At both depths, soil TC concentrations are significantly different in N-V from the other treatments (except for C-G at depth 2), with a mean of 156.66 mg C g^{-1} air dry soil compared to a treatment mean of 57.19 mg C g^{-1} air dry soil at depth 1; and a mean of 65.91 mg C g^{-1} air dry soil compared to a treatment mean (excluding C-G) of 46.72 mg C g^{-1} air dry soil at depth 2 (**Figure 4.14 A**). There were significant differences in soil TC concentrations between depths for each treatment except for C-F (**Figure 4.14 B**).

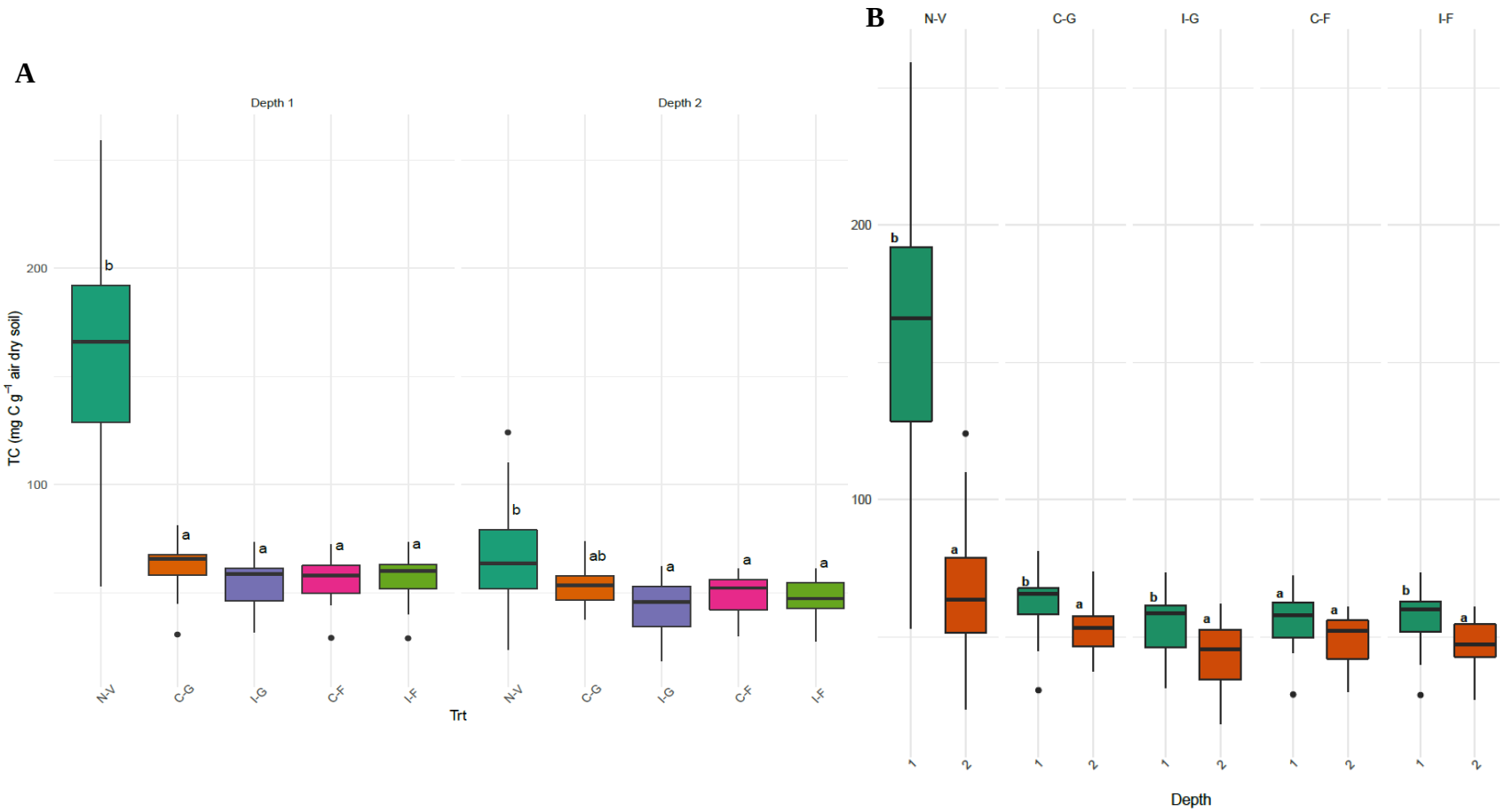


Figure 4.14. *Boxplots summarizing the significant differences in soil total carbon concentrations (TC; mg C g^{-1} air dry soil) among treatments described in* ***Table 3.1*** *at depth 1 (0-25 cm) and 2 (25-40 cm). In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Significant differences for the depth × treatment interaction across all sampling dates are represented in two ways: (A) differences in mean among treatments at each depth; and (B) differences in mean among depths for each treatments. Boxplots with the same letter code are not significantly different (Tukey HSD, $p < 0.05$).*

4.3 SOIL GENE TRANSCRIPT ABUNDANCE

The absolute transcript abundance of all the genes quantified varied within treatments as well as throughout the sampling dates and depths (**Figure 4.15**, **Figure 4.16**, and **Figure 4.17**). The dynamic changes in all gene transcript abundances were for the most part significantly influenced by time (**Table 4.5**). For both depths and C-G, I-G and C-F treatments, there were either small increments in gene transcript abundance from October to December/February or a stabilisation in gene transcript abundance from October to December/February; an increase in transcript abundance for all genes from February to March; a decrease in gene transcript abundance in May; and a stabilisation in gene transcript abundance from May to June (**Figure 4.15**, **Figure 4.16**, and **Figure 4.17**). All gene transcript abundances at depth 1 for the N-V treatment increased between October to December, which was followed by a stabilisation in gene transcript abundance throughout the entire non-growing season; whereas at depth 2, all gene transcript abundances for the N-V treatment followed the same pattern as the C-G, I-G and C-F treatments (**Figure 4.15**, **Figure 4.16**, and **Figure 4.17**). The *nifH*, *nirK*, and *nirS*, gene transcript abundance remained stable throughout the non-growing season in the I-F treatments for both depths (**Figure 4.15 A-B,** and **Figure 4.17 A-D**), except for AOA- and AOB-*amoA*, *narG*, *napA* and *nosZ-1*. The latter steadily increased from December 2017 to the mid-winter months (February and/or March of 2018) then decreased from the mid-winter months to the spring months (May and/or June of 2018; **Figure 4.15 C-F**, **Figure 4.16** and **Figure 4.17 E-F**). The overall pattern found in the data however is as follows: a stabilisation in gene transcript abundance in December prior to FTCs but a decrease in soil temperature, an increase in gene transcript abundance in March during reoccurring FTCs and flux in soil temperature, and a decrease in gene transcript abundance in June after FTCs and an increase in soil temperature.

Table 4.5. *Three-way ANOVA output to test the significances of the depths, sampling dates, treatments and their interaction term for the differences in average gene transcript abundance quantified (**Table 3.4**) at depth 1 (0-25 cm) and 2 (25-40 cm).*

Factor	*nifH*	AOA-*amoA*	AOB-*amoA*	*napA*	*narG*	*nirK*	*nirS*	*nosZ-1*
Depth	<0.01	<0.01	<0.01	0.21	<0.01	<0.01	0.03	0.15
Month	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.53
Trt	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.02
Depth*Month	0.07	0.08	0.18	1.00	0.02	0.12	0.11	0.43
Depth*Trt	<0.01	<0.01	0.01	0.68	<0.01	<0.01	0.09	0.05
Month*Trt	<0.01	0.84	0.90	<0.01	0.57	0.05	0.17	0.80
Depth*Month*Trt	0.68	0.56	0.18	0.07	0.36	<0.01	1.00	0.59
n	171	171	171	171	171	171	171	171

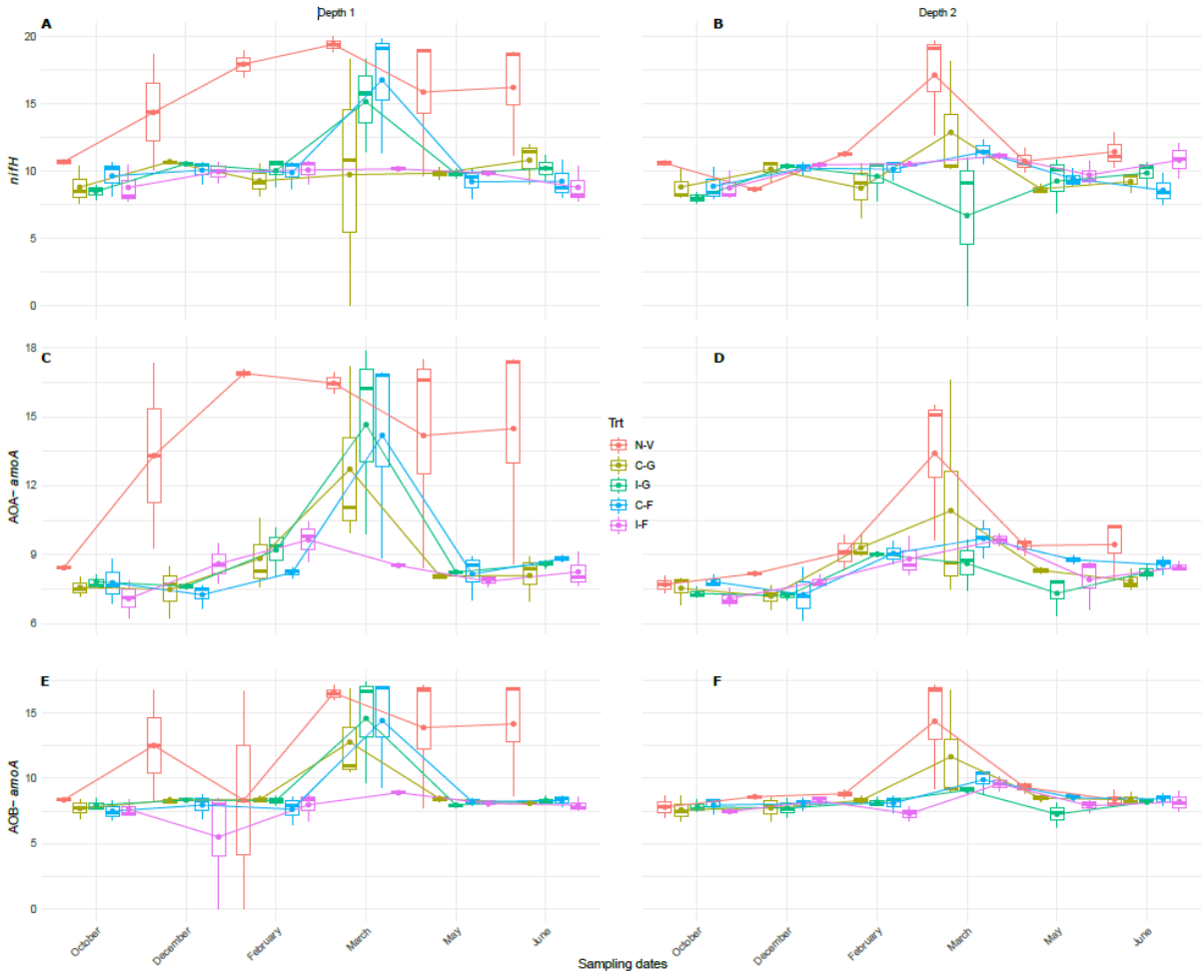


Figure 4.15. *Boxplots summarizing the: log_{10} transformed nifH (A and B), AOA-amoA (C and D) and AOB-amoA (E and F) gene transcript abundance among treatments described in* **Table 3.1** *at depth 1 (0-25 cm) and 2 (25-40 cm) for each sampling date. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Lines and points passing through the boxplots are monthly mean (n=6) values for each treatment.*

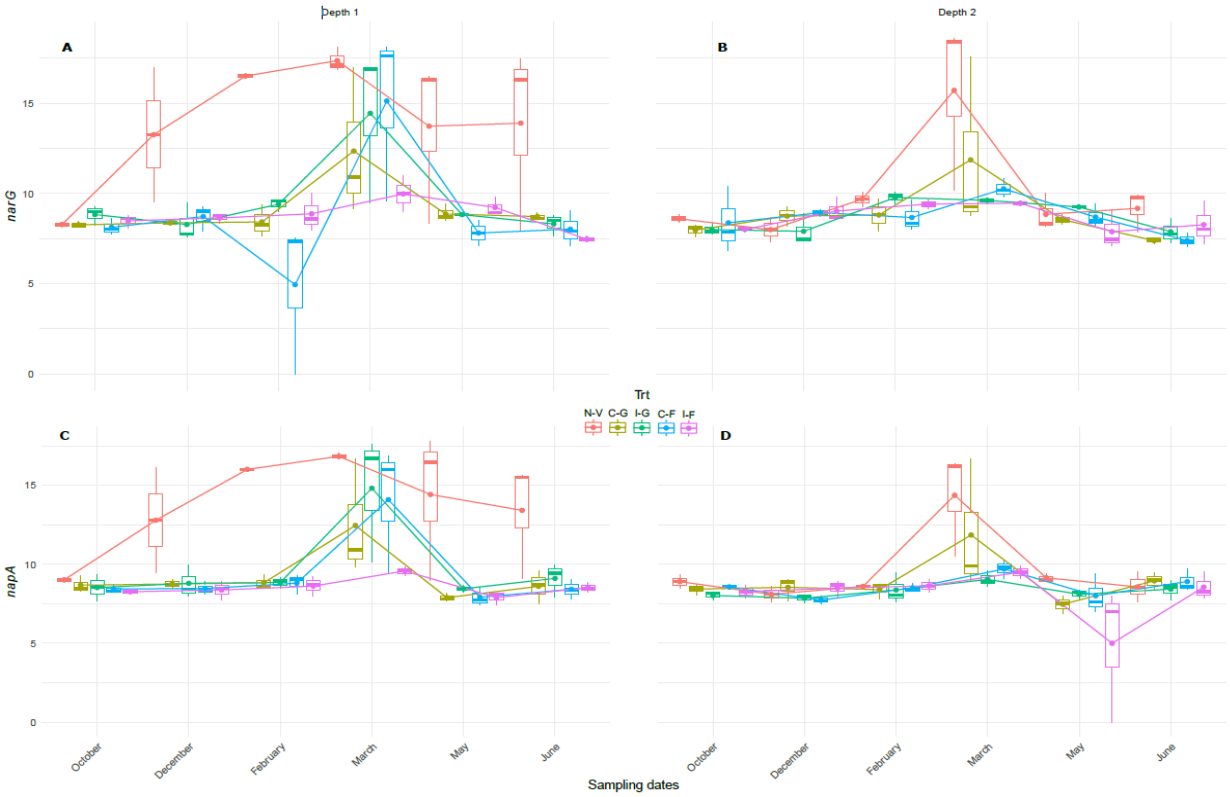


Figure 4.16 . *Boxplots summarizing the: log_{10} transformed narG (A and B) and napA (C and D) gene transcript abundance among treatments described in* ***Table 3.1*** *at depth 1 (0-25 cm) and 2 (25-40 cm) for each sampling date. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Lines and points passing through the boxplots are monthly mean (n=6) values for each treatment.*

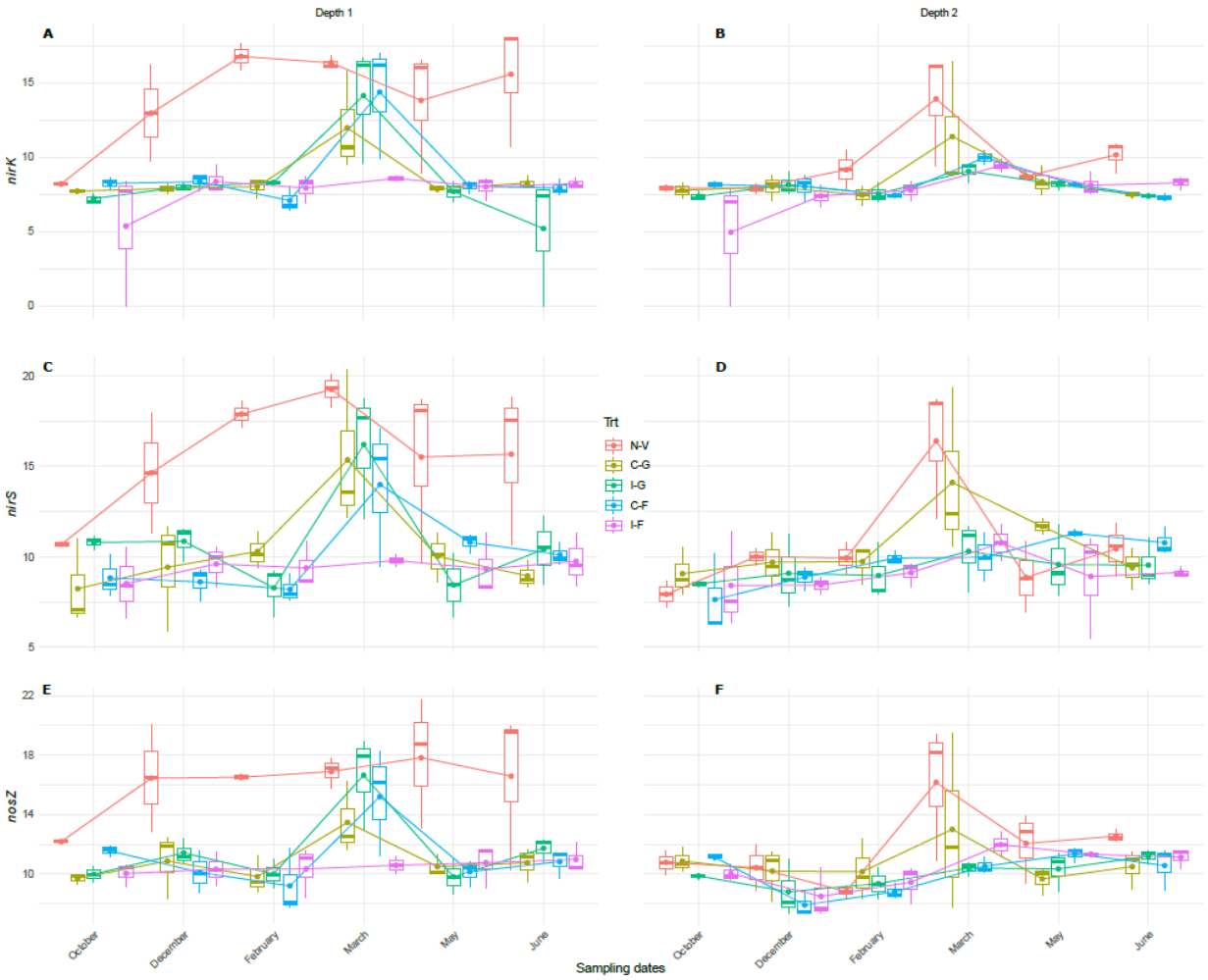


Figure 4.17. *Boxplots summarizing the: log_{10} transformed nirK (A and B), nirS (C and D) and nosZ-1 (E and F) gene transcript abundance among treatments described in* **Table 3.1** *at depth 1 (0-25 cm) and 2 (25-40 cm) for each sampling date. In each boxplot, the heavy horizontal line crossing the box is the median, the bottom and top of the boxes are the upper and lower quartiles, and the whiskers (if shown) are the max and min values. Lines and points passing through the boxplots are monthly mean (n=6) values for each treatment.*

4.3.1 *Winter gene expression exploratory analysis*

It was hypothesised that changes in all gene transcript abundance in agricultural soils exposed to cold conditions can be categorized by treatments and sampling time (Sections **2.6** and **3.7.2**). To test this, a linear discriminant analysis (LDA) was performed to determine how well the environmental profiles explain the *a priori* grouping (treatments in December, March and June, and between sampling dates in sections **2.6** and **3.7.2**). The two axes of the first three LDA models explain more than 84% of the dataset, neither of the gene transcript abundance profiles were able to classify and separate the treatment in each monthly grouping factors accordingly (**Figure 4.18 A-C**). However, the increase in AOA- and AOB-*amoA* and *napA* gene transcript abundance profiles were able to classify the March group (correlation coefficients of 71.3, 42.0 and 53.1 % with LD1 and -4.7, -25.1 and -23.3 % with LD2); the increase in *nosZ-1* and *nifH* gene transcript abundance profiles were able to classify the December group (correlation coefficients of 53.6 and 26.7 % for LD1 and -46.4 and 18.2 % for LD2); and the increase in *nirK*, *nirS* and *narG* gene transcript profiles were able to classify the June group (correlation coefficients of 55.3, 52.5 and 50.7 % with LD1 and -10.97, -45.9 and 13.1% with LD2).

Table 4.6. *Correlation coefficients describing the explanatory power for each gene transcript abundance profiles for the by month LDA.*

Variable	LD1	LD2
niFH	26.70%	-18.20%
AOA-*amoA*	71.27%	-4.70%
AOB-*amoA*	41.98%	-25.14%
narG	50.74%	13.10%
napA	53.05%	-23.34%
nirK	55.31%	-10.97%
nirS	52.50%	-45.93%
nosZ-1	53.57%	-46.38%

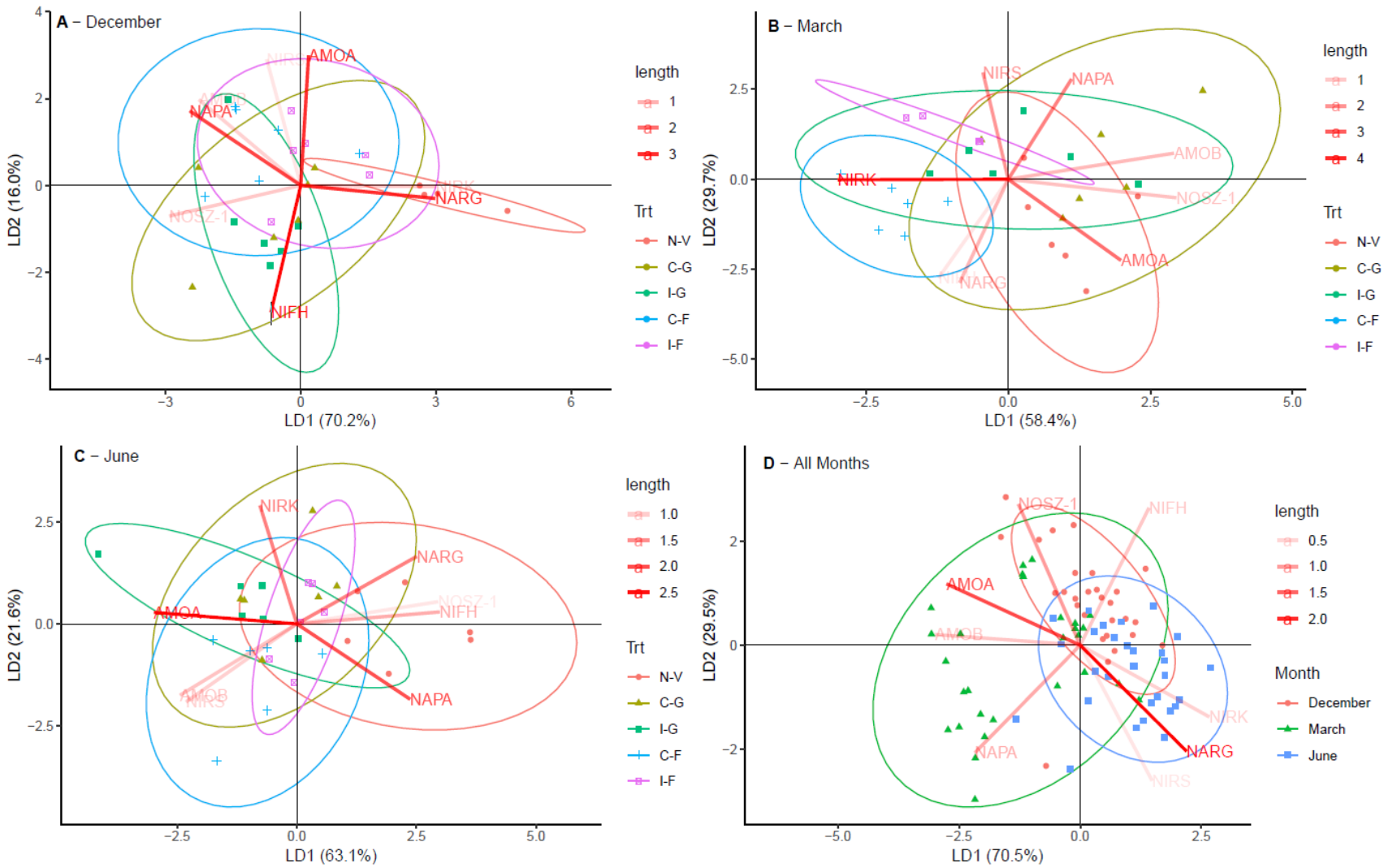


Figure 4.18. *Linear Discriminant Analysis biplots based on gene transcript abundances (***Table 3.4***) across both depths (A) December; (B) March; (C) June; (D) all months. While all variable arrows are of equal length, their relative contributions (scalings) are distinguished by colour gradients.*

4.3.2 *Relationships between environmental and N-cycling gene transcript abundance profiles*

To determine the relationships between gene expression abundance profiles, abiotic parameters, sampling dates and treatments a Canonical Correspondence Analysis was performed for both the sampling dates and treatments at each depth. The CCA sampling date and treatment models are both significant at depth 1 (*p*-value=0.001, **Table 4.7**). For both models at depth 1, CCA1 explains ≥62% of the variation in nitrogen cycling gene expression abundance profiles, while CCA2 explains ≥15% of the variation (**Figure 4.19 A** and **B**). In both the sampling date and treatment models (**Figure 4.19 A** and **B**), the *nifH*, AOA- and AOB-*amoA*, *narG*, *napA*, *nirK* and *nirS* gene expression abundance profiles are positively correlated to an increase in soil WFPS, pH, and NH_4^+-N concentrations but negatively correlated to a decrease in NO_3^--N and total carbon concentrations; whereas the *nosZ-1* gene expression abundance profiles are positively correlated to an increase in NO_3^--N and total carbon concentrations but negatively correlated to an increase in WFPS, pH, and NH_4^+-N. The variability in *nifH*, AOA- and AOB-*amoA*, *narG*, *napA*, *nirK* and *nirS* gene expression abundance profiles may be more likely explained by the soil conditions during the winter months (February and March), and less likely to be explained by the soil conditions during either the fall (October and December) or spring months (May and June); whereas the *nosZ-1* gene expression abundance levels are more likely explained by the soil conditions during the fall and spring months but less likely to be explained by the soil conditions during the winter months (**Figure 4.19 A**). The AOA-and AOB-*amoA*, *napA*, and *nirK* gene expression abundance profiles are more likely explained by the soil conditions in the N-V, I-G and I-F treatments, similarly the *narG*, and *nifH* gene expression abundance profiles are more likely to explained by the soil conditions in in the N-V, I-G and I-F treatments but may also include the C-F treatment (**Figure 4.19 B**). On the other hand, the *nosZ-*

1 gene expression abundance profiles are more likely to be explained by the soil conditions in the N-V, I-F and/or I-G treatments; whereas, the *nirS* gene expression abundance profiles are more likely to be explained by the soil conditions in the I-G and/or C-G treatments (**Figure 4.19 B**).

Table 4.7. *ANOVA output for the CCA sampling date and treatment models (n=85 each) at depth 1 (0-25 cm) and 2 (25-40 cm).*

CCA Model		F-Statistic	P-Value
Factor	Depth		
Month	1	16.16	0.001
	2	264.38	0.001
Treatment	1	36.69	0.001
	2	284.18	0.001

The CCA sampling date and treatment models are both significant at depth 2 (*p*-value=0.001, **Table 4.7**). For both models at depth 1, CCA1 explains ≥82% of the variation in nitrogen cycling gene expression abundance profiles, while CCA2 explains ≥16% of the variation (**Figure 4.19 C** and **D**). In both the sampling date and treatment models at depth 2 (**Figure 4.19 C** and **D**), the *nifH*, AOB-*amoA*, and *narG* gene expression abundance profiles are positively correlated to an increase in soil WFPS, TC and NH_4^+-N concentrations but negatively correlated to a decrease in NO_3^--N concentrations and pH; whereas the AOA-*amoA*, *nosZ-1*, *nirS*, *nirK* and *napA* gene expression abundance profiles are positively correlated to an increase in NO_3^--N concentrations and pH but negatively correlated to an increase in soil WFPS, TC and NH_4^+-N concentrations (**Figure 4.19 C** and **D**). All of the genes expression abundance levels are more likely to be explained by the soil conditions in December, March and February but less so in May, October and June (**Figure 4.19 C**); whereas all of the genes expression abundance levels are more likely to be explained by the soil conditions in the N-V, C-F and/or I-G treatments, but less so in the C-G and I-F treatments (**Figure 4.19 D**).

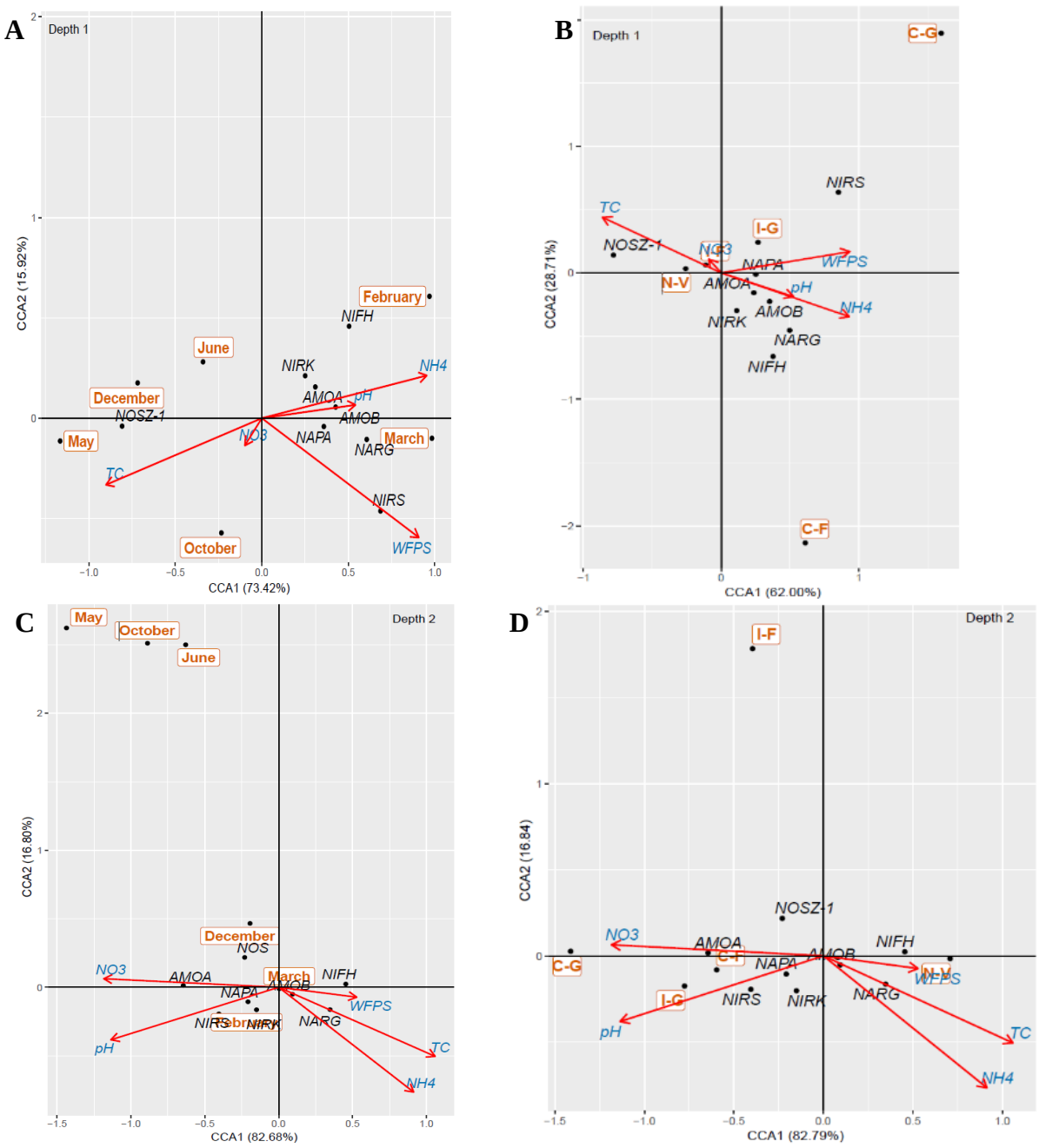


Figure 4.19. *CCA ordinations for the gene transcript abundance profiles using the (A) Sampling dates as objects at depth 1 (0.25 cm); (B) Treatments as objects at depth 1; (C) Sampling dates as objects at depth 2 (25-40 cm); and (D) Treatments as objects at depth 2. Objects are quantitative CCA factors that can be used to describe the probabilities that a certain response variable are most likely to be found or occur in. Variable used for the vector arrows were soil NH_4^+-N and NO_3^--N, total carbon concentrations (TC; µg/mg Element g^{-1} soil/ g^{-1} air dry soil), water-filled pore space (WFPS; %) and soil pH.*

4.3.2.1 Pearson correlations between N-cycling transcript abundance profiles with N-cycling genes, inorganic N and soil properties

To further test the associations between the N-cycling gene transcript abundance profiles and environmental parameters drawn from the CCA sampling date and treatment biplots at each depth, Pearson correlation matrices were performed for the entire dataset by depth (**section 3.7.2**). The models and matrices both provide significant biotic and abiotic evidence for the previously mentioned associations in **section 4.3.1** between the biotic and abiotic parameters (**Figure 4.20**, **Figure 4.21** and **Figure 4.22**). Across the entire dataset, i.e. at all sampling dates and treatments, there are significant positive correlations between all of the N-cycling gene transcript abundance profiles with one another, and soil NH_4^+-N and total carbon concentrations at depth 1 (**Figure 4.20 A**). However, there are significant negative correlations between the N-cycling gene transcript abundance profiles with NO_3^--N concentrations and pH at depth 1. There are little to no correlations between the N-cycling gene transcript abundance profiles with soil WFPS at depth 1 (**Figure 4.20 A**). At depth 2, across all sampling dates and treatments there are significant positive correlations between all of the N-cycling gene transcript abundance profiles with one another, soil NH_4^+-N and total carbon concentrations and WFPS (**Figure 4.20 B**). There are significant negative correlations between the N-cycling gene transcript abundance profiles with pH at depth 2 when averaged across all sampling dates and treatments. There was little to no correlation between N-cycling gene transcript abundance profiles with NO_3^--N concentrations at depth 2 (**Figure 4.20 B**).

Similarly, the simple linear models show weak positive relationships between the *nifH* and AOA-*amoA* gene transcript abundance profiles with the soil NH_4^+-N concentrations (r^2 of 0.12 and 0.15 respectively at depth 1; r^2 of 0.25 and 0.35 respectively at depth 2; **Figure 4.21 A-D**); and the *narG* and *nirK* gene transcript abundance profiles with the soil NO_3^--N

concentrations (r^2 of 0.13 and 0.2 respectively at depth 1; r^2 of 0.037 and 0.018 respectively at depth 2; **Figure 4.21 E-H**). The strength of the relationships increases with depth for the *nifH* and AOA-*amoA* gene transcript abundance profiles with the soil NH_4^+-N concentrations (**Figure 4.21 A-D)**, but decreases with depth for the *narG* and *nirK* gene transcript abundance profiles with the soil NO_3^--N concentrations (**Figure 4.21 E-H**). On the other hand, strong positive relationships exist between the *narG* and AOA-*amoA* gene transcript abundance profiles (**Figure 4.22 A** and **B**); the *nirK* and *narG* gene transcript abundance profiles (**Figure 4.22 C** and **D**); and the *nosZ-1* and *nirK* gene transcript abundance profiles at both depths (**Figure 4.22 E** and **F**). Moreover, the strength of the relationship decreases with depth between the *narG* and AOA-*amoA*, the *nirK* and *narG*, and the *nosZ-1* and *nirK* gene transcript abundance profiles (**Figure 4.22 A-F**).

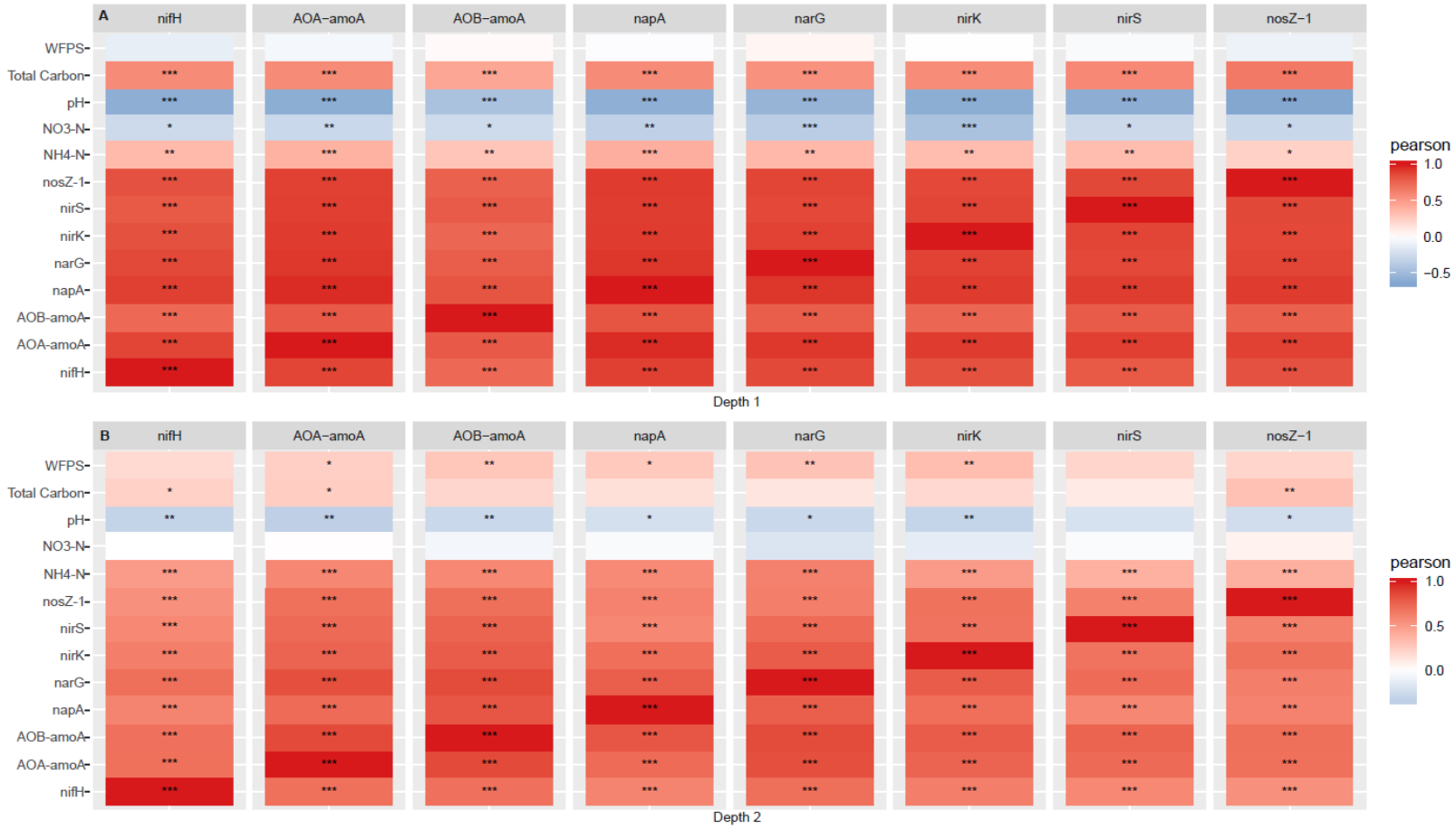


Figure 4.20. *Pearson correlation matrices showing the relative 1 to 1 significance and correlations between the N-cycling gene transcript abundance profiles, soil water-filled pore space (WFPS; %), pH, NH_4^+-N and NO_3^--N, and total carbon concentrations (TC; µg/mg Element g^{-1} soil/ g^{-1} air dry soil) at depth 1 (A, 0-25 cm; n=82) and depth 2 (B, 25-40 cm; n=85). Significance (p) is depicted by: * 0.05 to 0.01;,** 0.01 to 0.001; *** <0.001). The colour-coded scale represents Pearson rho (r), which is indicated by a positive (red) or negative (blue) correlation. Response and independent variables that are matched with one another (e.g. AOA-amoA and AOA-amoA) are always correlated and can thus be ignored.*

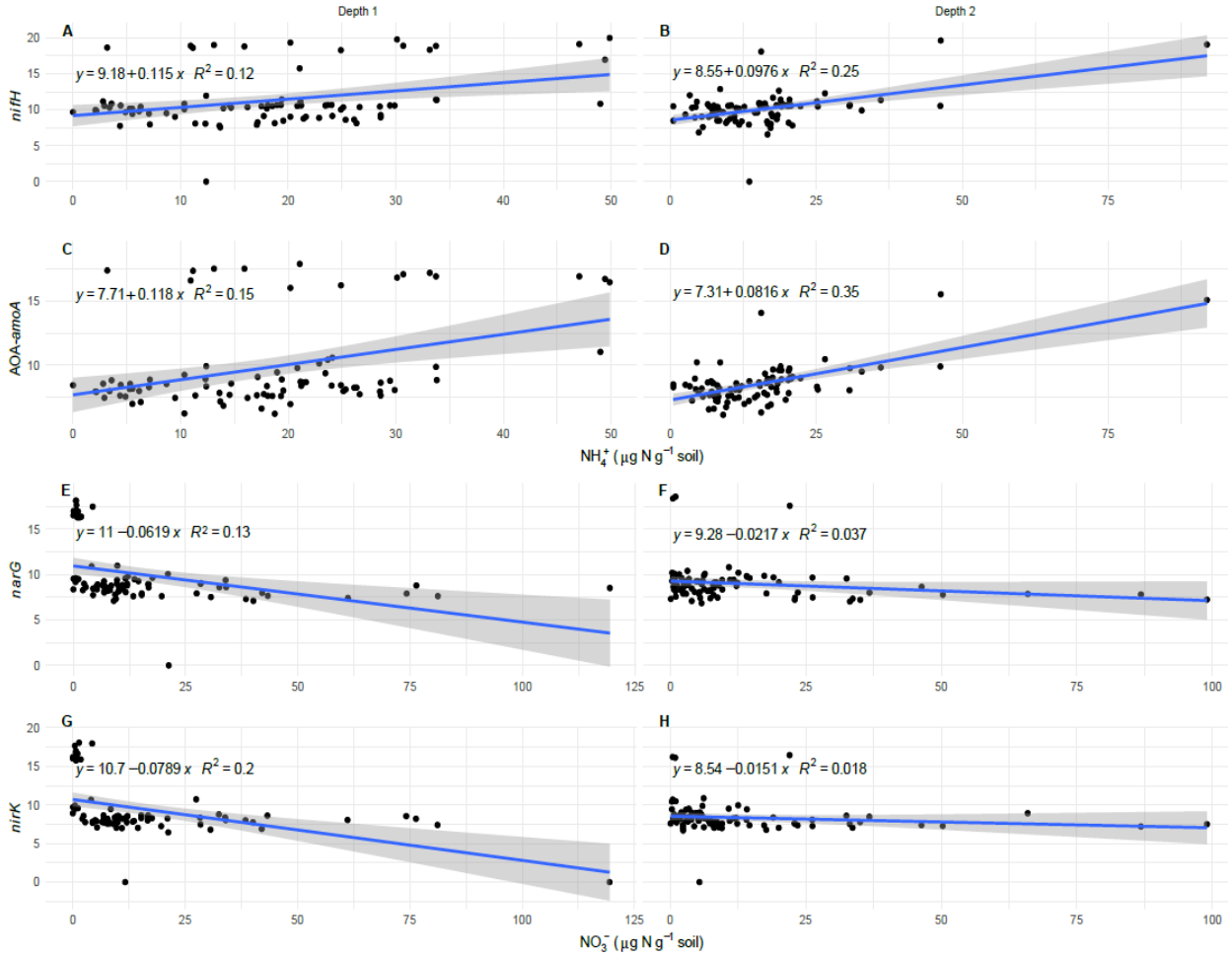


Figure 4.21. *Scatter plots depicting the relationship (r^2) between the soil NH_4^+-N or NO_3^--N, (µg N g^{-1} soil) and the log_{10} transformed nifH (A and B), AOA-amoA (C and D), narG (E and F), and nirK (G and H) gene transcript abundance.*

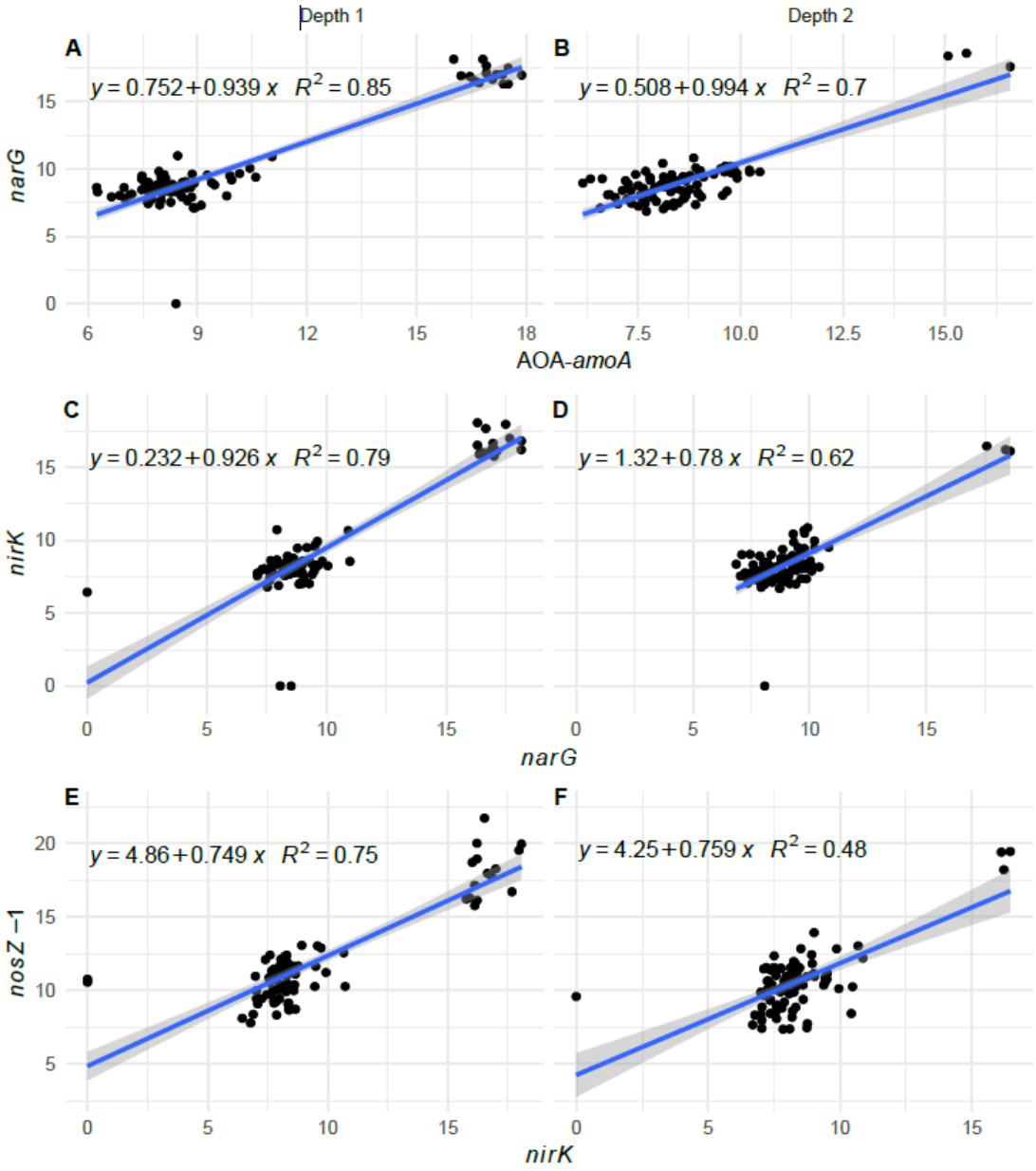


Figure 4.22. *Scatter plot depicting the relationship (r^2) between the AOA-amoA and narG (A and B), narG and nirK (C and D), and nirK and nosZ-1 (E and F) log_{10} transformed gene transcript abundance.*

5 DISCUSSION

5.1 ENVIRONMENTAL CONDITIONS OVER WINTER

Understanding the temporal dynamics of soils in boreal agricultural systems is essential as a shift of agricultural climatic conditions into high latitude areas like the boreal forest is projected (King et al., 2018). Moreover, climatic warming is predicted to also increase the frequency and amplitude of mid-winter warm spells and FTCs in high latitude areas which are linked to near 0 °C air temperatures, shallow snowpack and cyclic snowmelt, and soil freezing (Wertz et al., 2016; Wipf et al., 2015). Soils in the tested boreal agricultural system experienced repeated FTCs as early as December and persisting until mid-April, including recurring snowpack melting. However, soil temperatures within the plough layer (12 cm) during the non-growing season were consistently close to or above 0 °C, likely due to the thermal insulative properties of the vegetative cover present during winter (Congreves et al., 2018).

5.2 WINTER EFFECTS OF SOIL/CROP TREATMENTS ON SOIL PROPERTIES (WFPS, PH, TC, NO_3^-- AND NH_4^+-N)

5.2.1 *Soil WFPS*

Soil's bulk density, the related porosity and its capacity to retain water, as expressed in the WFPS measurements, were clearly affected by the land-use. Cropping increased porosity in the top layer but reduced it in the deeper soil layer. Nevertheless, there were variations in the soils capacity to retain water among the cropped treatments. The deeper rooting alfalfa increased overall porosity across the sampled C-F soil profiles and thus likely reduced the chances for ponding during winter snowmelt events (Westphal et al., 2018). Moreover, a consistent soil frost layer in the cropped plots enhanced the apparent water storage in the soil surface layer from mid-

December through mid-April. This was different from the natural condition where a deeper snowpack might have limited the formation of a soil frost layer. The subsoil layer had consistent water storage capacities across all tested conditions. These observations are in line with Congreves et al. (2018), which suggested that a frozen soil layer is favoured in the absence of a snow or vegetation cover, and can reduce water infiltration and soil hydraulic conductivity. As the frozen layer starts thawing the water accumulated at the soil surface and in the topsoil infiltrates into the deeper soil layers in a convective flow akin to flows induced by rain.

5.2.2 *Soil pH and inorganic N (NH_4^+ and NO_3^--N)*

Agricultural land-use, that in the region commonly involves repeated application of limestone, was reflected in an increased pH across the tested depth (Paul, 2007). On the other hand NH_4^+-N was found in larger concentration in the deeper soil layer under the forested plot, which is likely an indication of transport of the enhanced mineralisation in the organic rich upper soil layer. The concentrations of the more mobile NO_3^--N were very low in both layers of the natural plot. These observations thus hint at the likelihood for continuous mineralisation during winter in the forest soil (Campbell et al., 2014). Moreover, it might be speculated that excess protons associated with the oxidation of NH_4^+-N may have been a source for decreased soil pH. This may be assumed given that the soil WFPS remained <60% in the forested plots, ideal conditions for mineralisation in line with previous reports (Németh et al., 2014; Tatti et al., 2017). Previous studies have also shown that soil FTCs, the snowpack depth, fertilizer sources and plant material increase and release soil inorganic N during the non-growing season (Maljanen et al., 2009; Tatti et al., 2014, 2013; Westphal et al., 2018; Wipf et al., 2015).

In the agricultural land-use plots the soil pH, NH_4^+-N and NO_3^--N were statistically similar across treatments. A significantly higher soil NH_4^+-N was measured in the surface layer

versus the deeper layer for the I-G treatment, which included alfalfa and a late fertiliser application in September. Moreover despite minor differences among each other, the agricultural land-use plots all had higher soil pH and greater soil NH_4^+-N and NO_3^--N accumulation in the upper soil layer than the forested plots, a likely effect of the combination between N from fertiliser additions and mineralisation and the restrictive surface frozen layer. As mentioned above the latter was less significant for the forested soils that had a persistent deeper snowpack throughout the winter. Snowpack has been shown to mitigate freeze-thaw effects on nutrient fluxes (Wipf et al., 2015). Previous studies have shown consistent N release and loss is a result of the plant species' hardiness to the winter season frost, with the use of grasses, fescue and alfalfa typically having low to moderate soil NH_4^+-N and NO_3^--N loss (Maljanen et al., 2009; Westphal et al., 2018).

5.2.3 *Soil TC*

As expected in a relatively immature boreal forest with significant accumulation of carbon in its LFH horizon (Bitton, 2002; Paul, 2007) its soil TC concentrations were larger compared to the agricultural land-use plots. On the other hand, all agricultural land-use plots had statistically similar TC concentrations. Moreover, the top, plough, soil layer accumulated more carbon than the deeper soil layer. The exception was the C-F treatment where both soil layers had similar TC contents, a possible effect of the deeper rooted alfalfa. Previous studies have shown that soils in ecosystems with intermittent shallow/deep snowpack and naturally occurring FTCs, e.g. alpine or boreal regions, are more resistant to freeze-thaw induced nutrient leaching than from soils with historically persistent deep snowpack (Wipf et al., 2015).

5.3 TEMPORAL DYNAMICS OF SOIL PROPERTIES (WFPS, PH, NH_4^+- AND NO_3^--N, AND TC)

In the agricultural land-use soils there was an early release of NH_4^+-N in December, which coincided with the first snowfall and drop in air temperature of the winter. Soil NO_3^--N concentrations peaked in March, which coincided with a period of snow accumulation and air temperature above or close to 0 °C. Several studies suggested that the decrease in bioavailable N may be due to the deacclimation and damage of plant roots and/or microbial cell lysis that is subsequently leached with the snowmelt during FTCs (Campbell et al., 2014; Isobe et al., 2018; Joseph & Henry, 2008; Kreyling et al., 2015; Wipf et al., 2015). The forested soil NO_3^--N was consistently lower than in the agricultural land-use plots throughout the winter, and only increased with an increase in air/soil temperatures. In May, as the weather warmed the forested soil had NO_3^--N concentrations that were higher compared to the agricultural treatments, even if not always statistically significant. This suggests that the unique snowpack state, as mentioned above, and large amount of mineralizable leaf and litter residues might have led to losses of NO_3^--N via leaching with the movement of periodic snowmelt through the soil profile (Campbell et al., 2014; Kreyling et al., 2015; Wipf et al., 2015). This is supported by the correlation between soil NH_4^+-N and NO_3^--N concentrations and the soil WFPS status; a consistent but significant increase from December to February/March, a sudden decrease in March/May, followed by another increase from March/May to June. The consistent decrease in soil pH, coinciding with the trends in soil NO_3^--N concentrations, confirm previous reports that mineralization and denitrification activities occur in parallel (Németh et al., 2014; Wertz et al., 2013; Paul, 2007).

Winter soil TC also coincided with the trends in soil WFPS. Previous studies have shown that consistent soil C release and loss is mostly dissolved organic C (DOC) which is a result of either *de novo* carbon mineralisation of plant material and fine roots and/or microbial cell lysis (Campbell et al., 2014; Isobe et al., 2018; Kreyling et al., 2015; Wipf et al., 2015) governed by temperature and soil moisture (Tatti et al., 2017). On the other hand, in the forested plots the TC concentrations increased during the onset of snowfall and air/soil temperatures. While frequent FTCs at the early onset of snowfall in the winter can increase the soil DOC (Isobe et al., 2018), persistent winter deep snowpack accumulation and the subsequent large volumes of percolating water during recurring winter warm spells and FTCs is related to lower nutrient loss (Wipf et al., 2015).

5.4 WINTER EFFECTS OF SOIL/CROP TREATMENTS ON SOIL N-CYCLE GENE TRANSCRIPT ABUNDANCE

It was hypothesised that the dynamics changes in N-cycling gene transcript abundance profiles before, during and after winter might be linked to differences among soil/crop management regimes typical of Newfoundland agriculture. Moreover, it was also hypothesised that the transcript abundance profiles of the major genes (*nifH,* AOA- and AOB-*amoA, narG, napA, nirK, nirS,* and *nosZ1*) quantified may be used as a proxies for the potential N-cycling processes (N-fixation, nitrification or denitrification) and thus N-speciation. As such, the results of this project provides evidence that N-fixation, nitrification and denitrification were all co-occurring in the winter periods specific to the Maritime boreal ecozone in eastern Newfoundland. The nitrification and denitrification relevant genes have been previously reported to be expressed in soils during FTCs and have been used to provide evidence for the winter cycling of soil N

(Németh et al., 2014; Tatti et al., 2015, 2017; Yergeau & Kowalchuk, 2008). To my knowledge this is the first study to asses the *nifH* gene transcription abundance over the winter.

The treatment within the December, March and June sampling dates LDA models were able to explain ≥80% of the variance in the N-cycling gene transcript abundance profiles. However, this still did not allow to clearly discriminate among the treatment within the December, March and June sampling dates. This could be due to the relative similarities (i.e. crop species and fertilizer sources) among the tested agricultural land-use regimes which led to small temporal differences in the soil environmental variables (pH, WFPS and TC, NH_4^+-N and NO_3^--N concentrations) driving the gene expression abundance levels, a conclusion reinforced by previous reports (Tatti et al., 2014, 2017). In general, the N-cycling gene transcript abundance profiles were stable during the winter with a temporary increase in March. Exceptions were the treatment with the alfalfa and mineral fertilizer mix (i.e., the improved-forage treatment, I-F) and the natural plots. For the soils in the I-F treatment the gene transcript abundance was consistently low throughout the experimental period, with no notable increase at any time. The soil surface layer of the N-V control showed consistently high transcript abundances throughout the experimental period.

March was marked by an increase in soil water content and available nutrients, a common result of snow melting and of the nutrient and DOC availability due to the rapid mineralisation of the over-winter, FTCs induced, accumulation of physically fragmented and damaged plant tissues, and soil microbiota death (Campbell et al., 2014; Isobe et al., 2018; Joseph & Henry, 2008; Kreyling et al., 2015; Wertz et al., 2013). The low transcript abundance in the I-F treatment may be due to the availability and quality of the C and N within the alfalfa of this agricultural land-use regime that was unable to sustain N-fixers and (de)-nitrifiers over-

winter, an inference based on a previous study (Tatti et al., 2017). This might be explained by the comparable inorganic N and TC concentrations, and similar snowpack conditions among all agricultural land-use plots. The high transcription activity in the soils of the N-V treatment may be linked to the consistent snowpack presence and stable aerobic status (i.e., WFPS ≤60%) for these soils which may have allowed for sustained stable conditions and a constant release of inorganic N and TC. Winters without a snow cover and frequent freeze-thaw cycles have been shown to reduce the abundance of nitrifying (*Bacteria-amoA*) and denitrifying genes (*nirK*, *nirS* and *nosZ*) (Tatti et al., 2015; Su et al. 2010). Moreover, the input of residual plant material early, at or before the start of the winter can provide a steady release of organic C and inorganic N for the soil microbiota (Tatti et al., 2017).

5.1 TEMPORAL DYNAMICS OF N-CYCLE GENE TRANSCRIPT ABUNDANCE

As mentioned, most genes quantified were stable, but showed activity, before and after the March increase. Soil microbial communities have the capacity to adapt to winter conditions through changes in community structures (Zhang et al., 2014) during the fall to winter periods. Stable nitrite ammonifier and denitrifier abundance in soils subjected to manure or inorganic nitrogen amendments were previously reported and linked to cold-adapted microbiota that are capable of rapidly replacing not-adapted microbes (Tatti et al., 2014, 2017). The increase in N-cycling gene transcript abundances in the N-V soils, which increased after October and then remained stable throughout the winter, might be interpreted as clear evidence among treatments of such microbial community successional, adaptive shift. The increase in N-cycling gene transcript abundances for most of the agricultural land-use plots during an increase in the snowpack and for stable air temperatures above or close to 0 °C is evidence of the presence of an

adapted community that could react rapidly to any favourable shift in abiotic conditions (e.g. WFPS, pH), including an increase in DOC and inorganic N (Isobe et al., 2018). The decrease in expression during the second intense period of FTCs suggests FTCs induced microbial death and that even cold adapted microbiota were still susceptible to FTCs (Wertz et al., 2013). The unique behaviour of the gene expression in the alfalfa and mineral fertilizer treatment (I-F) suggests differentiation in either microbial community adaptation processes and/or differentiation in nutrient and soluble carbon availability and uptake rates by both plant and soil biota. This is a hypothesis to be further investigated.

5.1 RELATIONSHIPS BETWEEN SOIL BIOTIC AND ABIOTIC PARAMETERS DURING WINTER

The gene transcript abundances followed the temporal shifts in soil abiotic parameters. Winter (i.e., December, February and March) soil surface layer transcript abundances for all genes, except the nosZ-1, were directly correlated with the WFPS, pH and NH_4^+-N. The transcript abundance of *nosZ-1* gene was correlated to the concentrations of TC and NO_3^--N. While there were some variations among treatments, the correlative trends were found both in the natural and the agricultural-use plots. These correlations, which were specifically tested for each treatments and sampling dates, were stronger for the N-fixing and denitrifying genes transcript abundances than for the nitrifying genes transcript abundances It was evident that the soil saturation status was of importance. These observations are in line with previous reports that the increase in gene transcript abundance indicates the N-fixing, nitrifying and denitrifying microbial communities are likely responding to the increase in soil inorganic N and TC supply to continue microbial growth during favourable shifts in soil environmental conditions (WFPS and pH) (Chen et al., 2019; Hai et al., 2009; Y. Li et al., 2017; Liu et al., 2019; Samad et al., 2016;

Su et al., 2010; Sun et al., 2015). To my knowledge this is the first study to correlate the *nifH* gene transcript abundance with soil WFPS, pH and inorganic N and TC supply, and its implications on the active N-fixing microbial community over the winter.

5.2 RELATIONSHIPS BETWEEN SOIL BIOTIC AND ABIOTIC PROFILES

There significant positive biological relationships between the feedstocks (TC) and NH_4^+-N with all N-cycling gene transcript abundance profiles, but significantly negative relationships with soil pH. Moreover, there were strong correlations among the N-cycling gene transcript abundance profiles. A closer look has shown that the relationships between the availability of NO_3^--N and soil WFPS with all the N-cycling genes transcript abundance varied with soil depth. This was generally linked to a higher activity in the top layers, with more organic matter and thus more chances for nutrient availability from organic matter degradation. Furthermore, the surface soil layers are responding more readily to weather parameters. High nitrogen and carbon availability and a wet but not saturated (i.e., water filled soil porosity of ≤56% for nitrifiers or ≥60% for denitrifiers; Braker & Conrad, 2011) slightly acidic soil do favour the activity of N-cycling microbiota (Chen et al., 2019; Nicol et al., 2008; Samad et al., 2016; Wang et al., 2017), and additionally, the expression of genes along the N-cycling often occurs simultaneously (Németh et al., 2014; Tatti et al., 2017; Wertz et al., 2016). Similar to Tatti et al. (2017), this suggests that agricultural land-use regimes, such as crop species, fertilizer sources, amounts and application timing, provide selective hotspots and hot moments for the transcriptional activity of the N-fixing and (de)-nitrifying genes through the minute modifications of environmental parameters over-winter. However in contrast to Tatti et al. (2017), it may be hypothesised that any FTCs driven variability in the soil environmental parameters creates short-term windows for

ideal ranges that govern the winter shifts in N-cycling gene transcript abundance and activity. Co-occurrence of favourable abiotic conditions and the co-expression of the various N-cycling genes are both necessary and expected.

The results support the hypothesis that gene expression along the N-cycle chain co-occur and are correlated to the soil TC and NH_4^+-N, offering support to the fact that the metabolism of nitrogen and carbon are proximately related. Moreover, the gene-to-gene models were statistically more significant ($r^2 \geq 0.48$) than the gene-to-feedstock models ($r^2 \leq 0.35$). It is however, emphasized that lacking actual enzymatic assays while the relationships may be reasonably assumed, only further gene expression-enzymatic-activity-feedstock/product experiments may offer full support to the elucidation of the gene expression to feedstock relationship hypothesis. Still, previous reports have shown that the inherent activity (i.e. paired C and N metabolism) of the functional ammonia oxidizing microbial communities have the potential to stabilize NH_4^+- and NO_3^--N availability, through the upregulation of *amoA-hao* mediated nitrification pathway (Lamba et al., 2017; Norman et al., 2015). Nevertheless, it is here argued that such an endeavour is necessary and likely. Moreover, inclusion of a full N and C speciation analyses would be recommended. This may then link information in this experiment with known reports of the correlations between enzymatic activities and related to soil N emissions (Isobe et al., 2018; Liu et al., 2019; Qin et al., 2018; Samad et al., 2016; Tatti et al., 2017).

TC and NH_4^+-N were linked in a direct relationship with the *nifH* transcript abundance. Soil pH had an inverse relationship with the *nifH* transcript abundance. The *nifH*'s putative relationship to WFPS and the negative relationship with NO_3^--N concentrations depended on depth. While C and available soil water favour upregulation of *nifH,* increases in soil pH and

bioavailable N, including NO_3^--N, are related to the downregulation of *nifH* (Chen et al., 2019; Pereira e Silva et al., 2013). The results of this experiment confirmed that these relationships are also relevant to the *nifH* gene transcript abundance profiles during the cold, winter season.

6 CONCLUSION

To my knowledge this is the first study to validate that the main N-fixing and (de)-nitrifying genes (*nifH*, AOA- and AOB-*amoA*, *narG*, *napA*, *nirK*, *nirS* and *nosZ*-1) were expressed during winter in agricultural fields in the Atlantic Boreal Maritime ecozone. These findings offer support to the assumption that N-fixation and (de)-nitrification co-occur in winters of this region. While the impact of crop production was marginal there is evidence that alfalfa might enhance the expression of N-cycling genes downstream from ammonification. Land-use had the strongest impact on N-cycling transcript abundance with significant decreases in the agricultural plots vs. the forested plots. Early spring warming and snow melting led to overexpression of all N-cycling genes. Moreover, the presence of a snowpack was most important for increasing the N-fixing and (de)-nitrifier gene transcript abundance. Furthermore, winter expression of the N-fixing and (de)-nitrifying microbiota responds to short-term changes in environmental parameters. The presence of a pure alfalfa plant community with mineral fertilizer as an N source was linked to the lowest gene transcript abundances, suggesting a more tightly balanced nutrient cycle and possibly a lower risk for N losses. The expression of each N-cycling gene was best explained by the expression of all other N-cycling genes. Freeze-thaw during winter creates short-term windows when the abiotic parameters are in ranges favourable to the expression of N-cycling genes. The lower statistical significance of the relationships between gene expression and feedstock availability (i.e. NH_4^+-N and NO_3^--N) may be expected given that gene expression is not necessarily related with the activity of the translated enzymes. The inconsistencies needs to be elucidated in a time-series, controlled experiments, that verify all components of the abiotic trigger – gene expression – enzymatic activity feedback loop, including N species along the N-cycle. Moreover, the results have shown that the winter

expression of the *nifH* gene was negatively correlated to the soil pH and NO_3^--N, suggesting that *nifH* gene is downregulated, probably in order to balance microbial growth in acidic soil conditions.

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8 SUPPLEMENTARY INFORMATION

Figure 8.1. *Soil surface conditions for treatments 0-4 (A) during the February sampling date; (B) after the February sampling date, and (C)during mid-March of 2018.*

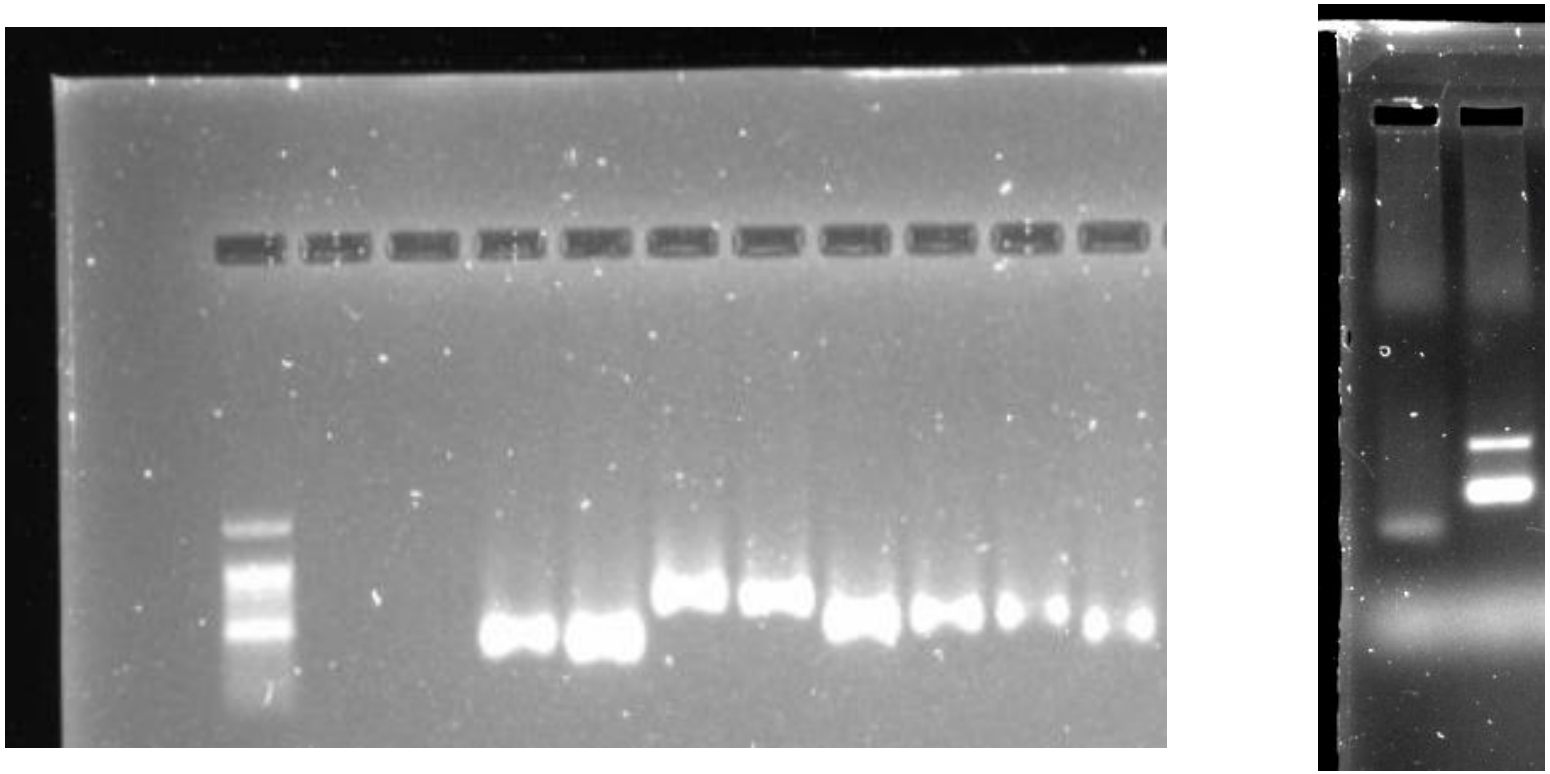

Figure 8.2. *Gel electrophoresis validation for N-cycling gene primer sets. Primer pairs are from left to right, nifH, AOA- and AOB-amoA, narG, napA, nirK, nirS and nosZ-1, and Archaea- and Bacteria 16S.*